FATTY ACIDS IN COD LIVER OIL

Livinus Ariri Donatus Duru

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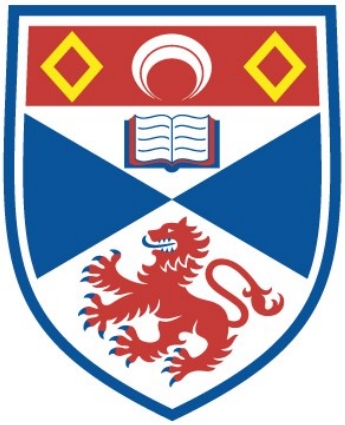
at the
University of St Andrews



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DECLARATION

I hereby declare that this thesis is a record of the results of my own experiments, that it is my own composition, and that it has not previously been presented in application for a higher degree.

The research was carried out in the Department of Chemistry, United College of St. Salvator and St. Leonard, University of St. Andrews, under the supervision of Professor F.D. Gunstone, D.Sc., FRIC.

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CERTIFICATE

I hereby certify that LIVINUS ARIRI DONATUS DURU has completed twelve terms of research work under my supervision, has fulfilled the conditions of the Resolution of the University Court 1967, No. 1 (St. Andrews) and that he is qualified to submit the accompanying thesis in application for the degree of Doctor of Philosophy.

Professor F.D. Gunstone
(Research Supervisor)

UNIVERSITY CAREER

I completed my M.Sc. degree at the United College of St. Salvator and St. Leonard, University of St. Andrews in April 1977.

I was admitted as a Ph.D. student in the United College, University of St. Andrews in September 1977.

I was supported by the Federal Government of Nigeria in 1977-78, by the Science Research Council in 1978-79, and by the Department of Chemistry, University of St. Andrews in 1979-80.

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ABBREVIATIONS

Fatty acids are reported in shorthand notation by indicating the number of carbon atoms followed by a colon and a figure denoting the number of multiple bonds. The nature of unsaturation (shown by the letters c and t indicating cis olefinic and trans olefinic groups respectively) and its position relative to the carboxyl group is given in parenthesis.

The symbol Δ indicates the position of the double bond, counting from the carboxyl-end of the carbon chain and n-x shows the location of the double bond from the methyl end, where x is the position of the first double bond counting from the methyl-end of the molecule, e.g. 18:2 (n-6) is the same as 18:2 (Δ9,12).

Ag^{+}tlc	Argentation thin layer chromatography
CMR	^{13}C nuclear magnetic resonance
CE	cyclic ether
DMCS	Dimethylchlorosilane
DMF	Dimethylformamide
ECL	Equivalent chain length
F	Furanoid. Symbols F1, F2, F3 etc denote the furanoid acids commonly occurring in the fish lipids
FCL	Fractional chain length
FID	Flame ionisation detector
GC-MS	Gas chromatography-mass spectrometry
glc	Gas liquid chromatography
HMDS	Hexamethyldisilazane
HPLC	High pressure liquid chromatography
ir	Infrared
LAH	Lithium aluminium hydride

M^+	Molecular ion
ML	Mother liquor
MS	Mass spectrum or Mass spectrometry
NMIA	Non-methylene-interrupted polyene acid
nmr	Nuclear magnetic resonance
O-TMS	= Trimethylsilyloxy
P_{16}	Methyl 4, 8, 12-trimethyltridecanoate [4, 8, 12-TMTD]
P_{17}	Methyl 5, 9, 13-trimethyltetradecanoate [5, 9, 13-TMTD]
P_{19}	Methyl 2, 6, 10, 14-tetramethylpentadecanoate [2, 6, 10, 14-TMPD (pristanic)]
P_{20}	Methyl 3, 7, 10, 15-tetramethylhexadecanoate [3, 7, 10, 15-TMHD (phytanic)]
PE	Denotes a mixture of light petroleum (bp 40-60°) and diethyl ether. For example PE10 denotes 10% diethyl ether in light petroleum
PMR	Proton magnetic resonance (^{1}H nuclear magnetic resonance)
PUFA	Polyunsaturated fatty acid
THF	Tetrahydrofuran
THP	Tetrahydropyran
tlc	Thin layer chromatography
TMCS	Trimethylchlorosilane
TMS	Trimethylsilyl
TPP	Triphenylphosphine
uv	Ultraviolet

ABSTRACT

The isolation and structural identification of the minor components of cod liver oil was undertaken with particular attention to those of novel structure. A series of procedures based on urea crystallisation, argentation chromatography and preparative glc were developed for the isolation of the unusual acids present at low level. With these procedures, phytol-based acids (isoprenoids), furanoid acids and a series of mono- and diunsaturated branched-chain fatty acids were isolated. These fatty acids were characterised by a combination of mass spectrometry and nmr. In the case of the branched-chain acids, a GC-MS capillary system was used. Pyrrolidide and O-TMS derivatives of the acids were also studied for the location of double bonds.

Three phytol-based acids were identified. In addition to confirming the presence of nine furanoid acids, new furanoid acids were also discovered. The discovery of a branched diunsaturated acid is significant. The 7,9-dimethylhexadec -6,8-dienoic acid is novel and is reported in this study for the first time.

In addition to these acids, the major component acids of cod liver oil were isolated and identified. For the isolation of these, procedures such as low temperature crystallisation, argentation chromatography and preparative glc were adopted. Ozonolysis followed by reduction with triphenylphosphine was used for the analysis of the positional isomers of monoene acids. For diene acids ozonolysis was followed by reduction with sodium borohydride. The merits and demerits of these two procedures for the location of double bonds in a fatty acid chain are discussed.

Oxymercuration-demercuration was used for the characterisation of some of the polyene acids. Only polyene acids having Δ3t, Δ4 or Δ5 unsaturation react in this procedure to furnish cyclic ethers which can be isolated and identified. The fragmentation characteristics of these cyclic ethers are discussed.

INTRODUCTION

1.1 THE STRUCTURE AND BIOSYNTHESIS OF FATTY ACIDS WITH SPECIAL REFERENCE TO THOSE PRESENT IN FISH LIPIDS

1.1A Structure

a) General features of fish acid structures

The history of the fatty acid composition of fish oils is well documented and extensively treated, by many investigators[1-4]. Knowledge of the structure of fish oil fatty acids and their distribution in marine life is important for the development of fishery products and for the evaluation of the nutritional significance of fish oils.

Marine animal and fish lipids are characterised by fluidity at low temperature, by a wide range of saturated and unsaturated acids with a high ratio of n-3/n-6 acids, and by the frequent presence of fatty acids of unusual structure. Referring to the complexity of marine lipids, Ackman[5] described them as "the most comprehensive assemblages of fatty acids of all chain lengths, degree of unsaturation and isomeric composition which have presented a daunting problem to the lipid analyst".

Gunstone[6], in his assessment of the general characteristics of fatty acids from aquatic origin concluded:

(i) The fatty acids present in fish oils usually exceed twenty in number. They vary in chain length from C_{14} to C_{24} occasionally including C_{12} and C_{26} acids along with acids of odd chain length.

(ii) The saturated acids (25%) consist mainly of palmitic acid (15-20%) accompanied by smaller proportions of myristic and stearic acids.

(iii) Most fish oils contain several monoene acids (usually 35-60%). The C_{18} members (mainly Δ9 but sometimes with Δ11) predominate, followed by the C_{16} (Δ9), C_{20} (Δ9 and Δ11) and C_{22} (Δ11 and Δ13) acids.

(iv) Many C_{16}, C_{18}, C_{20} and C_{22} polyene acids are present. The 20:5 and 22:6 n-3 acids are generally the major components, accompanied sometimes by 22:5 and less commonly by 20:4 . The major polyene acids belong to the linolenic series and have the general formula:

$$CH_3.CH_2\lfloor CH{=}CHCH_2\rfloor_n\lfloor(CH_2\rfloor_m COOH$$

In addition to the general features common to fish oil fatty acids, the following characteristics have been observed. Fats derived from freshwater fish contain more C_{16} and C_{18} monoene acids and less C_{20} and C_{22} polyene acids than fats from marine fish. Marine animals (the dophin, porpoise, and other species of Delphinidae) are characterised by high proportions of the unusual isovaleric acid (C_5). Some fish oils, such as shark oil, contain large quantities (20-80%) of unsaponifiable material which is mainly the terpene, squalene, and some glyceryl ethers. Sperm whale oil consists mainly of ester waxes derived from long-chain saturated and monounsaturated acids and alcohols.

b) Saturated fatty acids

Saturated fatty acids usually amount to 25-30% of which palmitic acid (15-20%)[6] is the major component. Other even-chain acids from C_{12}-C_{26} may also be present. Odd-chain acids - mainly C_{15}, C_{17} and C_{19} - frequently total 1-2%, but occasionally they are at higher levels as in the mullet where they make up a quarter of the

saturated acids. The amphipod - Pantoporeia femorata - containing ~ 50% of odd-chain acids is probably an important source of these unusual acids in the food chain.

c) Monomethyl branched-chain acids

Branched-chain acids occur in fish lipids at a very low level. Iso- and anteiso- acids, with the additional methyl group on the penultimate and antepenultimate carbon atoms respectively, are widely distributed throughout the animal kingdom. The C_5 acid (isovaleric) for example is present in the jaw-bone oil of the dolphin and the porpoise whilst the iso C_{15} and anteiso C_{15} and C_{17} acids have been identified in shark liver oil.

Olefinic iso- and anteiso- acids which occur in marine diatomaceous ooze[7] have yet to be fully identified in fish lipids.

d) Polymethyl branched-chain acids

The polymethyl-branched acids present in most fish oils are derived from the C_{20} alcohol phytol. The most common acids are 3,7,11,15-tetramethylhexadecanoic (phytanic acid for which the abbreviation P_{20} is used in this report), 2,6,10,14-tetramethyl-pentadecanoic (pristanic acid, P_{19}) and 4,8,12-trimethyltridecanoic acid (P_{16}). These acids are widely distributed - though at a low level - and the ratios P_{16}/P_{20} and P_{19}/P_{20} have been claimed by Ackman and Hooper to be characteristic of their fish source.

The isoprenoid acids occur widely and P_{20} has been identified in ox perinephric fat[8], ox plasma lipids[9], milk fat[10,11], lipids of various animals[12,13] and fish oils[14]. Isoprenoid acids have also been isolated and identified from lipids of marine organisms[15-18] and in ancient[19,20] and recent geological sediments[21].

e) Furanoid acids

Unusual fatty acids containing a furan ring system have been isolated and identified in fish lipids[22-25], a seed oil[26], and recently from the rubber latex[27]. The structures of some of these acids have recently been confirmed by synthesis[28]. A fuller account of the structures of the furanoid acids and some new ones isolated and identified in this project are given in section 2.3 .

f) Monoene acids

Monoene acids present in fish oils range from C_{10}-C_{24} (even numbers) with the C_{14}-C_{22} members being the most important. They are generally present at a level of 30-50%. As already indicated fats from freshwater fish contain more of the 16:1 and 18:1 acids and less of the 20:1 and 22:1 acids with this situation frequently being reversed in the marine lipids. Minor amounts of odd-chain monoene acids have also been reported. The position of the unsaturated centre in these acids is discussed in section 2.5 .

g) Polyene acids

The polyunsaturated acids (PUFA) of fish oils include dienes (1-7% of total acids), trienes (1-6% of total acids), tetraenes (1-8% of total acids), pentaenes (9-15% of total acids) and hexaenes (2-14%). The total content of PUFA in fish oils varies from species to species. For example the total PUFA of alewife freshwater fish is 28% and that of Atlantic herring about 19%[29].

The characteristic methylene-interrupted double bond pattern is present in the majority of fish oil PUFA. These acids belong almost entirely to the n-6 and n-3 families with the latter generally predominating. The structures of these acids are indicated in the

following section on biosynthesis. In addition to the common C_{18}, C_{20} and C_{22} PUFA, others identified in marine species include a more highly unsaturated C_{18} acid 18:5 (3, 6, 9, 12, 15), the odd-carbon acids 17:4 (6, 9, 12, 15), 19:4 (5, 8, 11, 14) and 21:5 (6, 9, 12, 15, 18) and the C_{24} acids (Δ9, 12, 15, 18, 21 and Δ6, 9, 12, 15, 18, 21)[32].

Some non-methylene-interrupted acids have been identified recently in marine organisms. Two homologous series of C_{20} (Δ5, 11; Δ5, 13) and C_{22} (Δ7, 13; and Δ7, 15) acids were identified from a number of marine sources by Paradis and Ackman[30], and Ackman and Hooper[31]. Recently Pearce and Stillway[32] reported additional non-methylene-interrupted dienes in many organisms. These generally have a double bond in the Δ5 or Δ7 position and include the Δ5, 11; Δ5, 13, Δ7, 11; and Δ7, 13, C_{20} dienes and the Δ7, 13; Δ7, 15; Δ9, 13; Δ9, 15; and Δ7, 17 C_{22} dienes.

1.1B Biosynthesis

Fatty acids present in fish lipids may be wholly endogenous or wholly or partly exogenous. Fish usually have a fat-rich diet so that the amount of wholly endogenous material may be quite low. Indeed Cowey and Sargent[33] have recently suggested that some species of fish lost - or never acquired - the ability to form PUFA from C_{18} precursors as these abound in large quantities in their diet.

The following review of fatty acid biosynthesis is of a general nature covering plants and animals whether these exist on land or in water.

a) de novo synthesis

Fish, like all other forms of organisms, synthesise saturated straight-chain acids (even) de novo by the acetate-malonate pathway. Modifications of the usual pathway leading to other acids are achieved by using a different "primer" (iso-, anteiso- acids) or by replacement of malonate by methyl malonate to give methyl-branched acids.

The overall biosynthetic process can be depicted thus:-

$$CH_3COSACP + 7HO_2CCH_2COSACP \longrightarrow CH_3(CH_2)_{14}COSACP + 7CO_2$$

(a) (b) (c)

The "primer" molecule above (a) can be replaced by other acids to produce fatty acids of different chain lengths and structure. The molecule (b) is produced from acetate and CO_2 but in (c) all carbon atoms are acetate derived.

Alternative "primers" and the resultant acids are shown below.

$CH_3CO_2H \longrightarrow$ straight-chain even acids - mainly C_{16}

$CH_3CH_2CO_2H \longrightarrow$ straight-chain odd acids - mainly C_{17}

$$CH_3\underset{\displaystyle CH_3}{\underset{|}{C}}HCO_2H \longrightarrow \text{even iso acids, mainly } C_{18}$$

$$CH_3CH_2\underset{\displaystyle CH_3}{\underset{|}{C}}HCO_2H \longrightarrow \text{odd anteiso acids, mainly } C_{17}$$

b) Chain extension

Chain extension occurs with endogenous and exogenous saturated and unsaturated acids to produce fatty acids of different chain lengths. The main product of de novo synthesis (16:0) may undergo chain extension to give stearic acid and the process may be repeated to furnish fatty acids of still longer chain-length.

c) Desaturation

In fish the Δ9-desaturase is the main enzyme desaturating saturated acyl groups and leading to the production of Δ9c monoene acids. Chain-extension may then operate to give longer-chain monoene acids. The docosenoic acid (22:1, n-11) a common in marine lipids is mainly derived from monoethylenic fatty alcohols of wax esters in marine crustacean[34]. The biochemical conversion of fatty alcohols to fatty acids is responsible for the peculiarity that 22:1 acids of marine origin are predominantly cis Δ11 rather than cis Δ13. It is also possible that 22:1 (n-11) could be formed from the desaturation of 20:0 to 20:1 (n-11) by a Δ9-desaturase followed by chain-extension[33]. The 24:1 (Δ15c) (nervonic acid) originates from oleic acid via chain-extension of 22:1 (Δ13c) erucic acid. The biosynthetic pathway for the production of major monoenes via chain elongation and desaturation is shown below:

n-9 series: 18:0 ⟶ 18:1 (9) ⟶ 20:1 (11) ⟶ 22:1 (13)

n-11 series: 20:0 ⟶ 20:1 (9) ⟶ 22:1 (11)

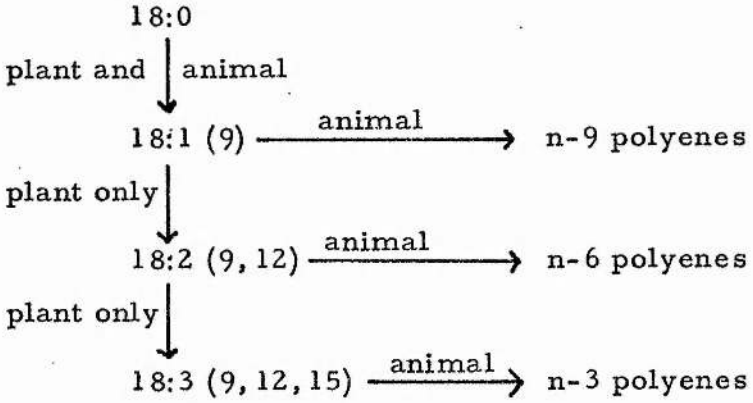
Three other desaturases (Δ4, 5 and 6) are involved in the production of polyenoic acids, giving both methylene-interrupted (see section e) and non-methylene-interrupted isomers. The Δ7, 13 C_{22} diene acid probably results from the following sequence:

18:0 $\xrightarrow{\text{9-desaturase}}$ 18:1 (9) $\xrightarrow{\text{chain-extension}}$ 20:1 (11) $\xrightarrow{\text{5-desaturase}}$ 20:2 (5, 11) $\xrightarrow{\text{chain-extension}}$ 22:2 (7, 13)

d) Polyenes resulting from plant desaturation

Plant desaturase systems are capable of biosynthesising 18:1 (9), 18:2 (9, 12) and 18:3 (9, 12, 15) from the 18:0 acid by successive desaturation. The animal systems can then produce the n-9, n-6,

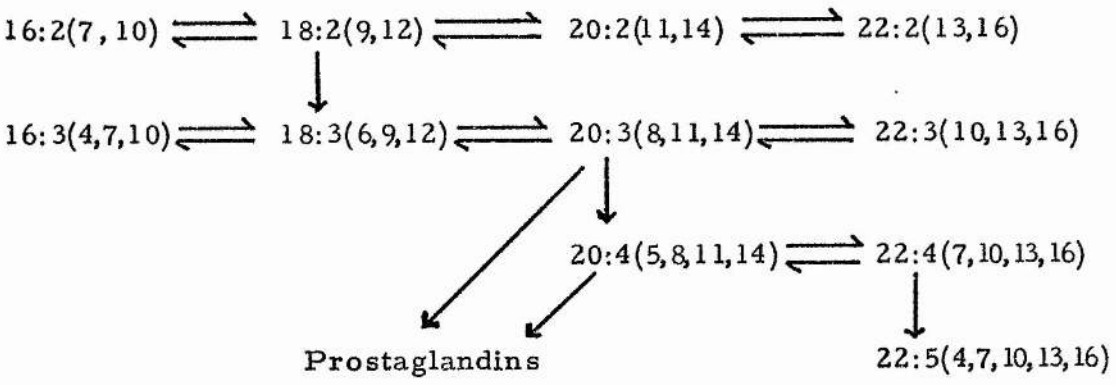
and n-3 families of polyene acids from the plant C_{18} acids by chain-elongation and desaturation. The relationship between the two systems (plant and animal) is shown below -



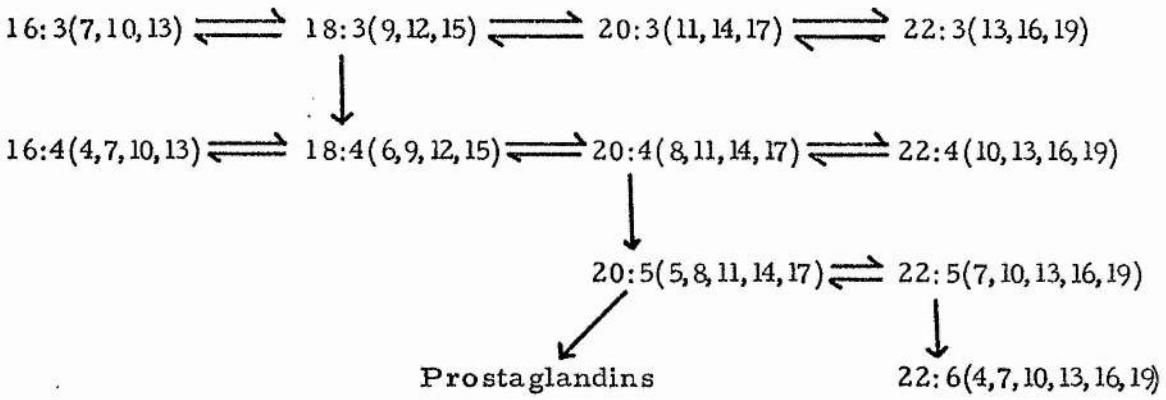
e) Polyenes resulting from animal desaturation

Fish, in common with other animals, modify dietary fatty acids to suit their own requirements. Notably, the fish do not contain a desaturase which can introduce a double bond beyond the Δ9 position of the monoene acids obtained by de novo synthesis. The abundant n-6 and n-3 polyene acids in fish may be dietary in origin or may be derived from C_{18} precursors of plant origin. The major pathways in polyene biosynthesis in animals (including the fish) is shown below.

n-6 series



n-3 series


16:3(7,10,13)
18:3(9,12,15)
20:3(11,14,17)
22:3(13,16,19)
16:4(4,7,10,13)
18:4(6,9,12,15)
20:4(8,11,14,17)
22:4(10,13,16,19)
20:5(5,8,11,14,17)
22:5(7,10,13,16,19)
Prostaglandins
22:6(4,7,10,13,16,19)

1.2 FATTY ACIDS IN COD LIVER OIL

1.2.1 Fatty acid structures

a) Saturated acids

The n-alkanoic acids of cod liver oil constitute 14-16% of the total acids[17]. These include the even acids: C_{12} (0.1%), C_{14} (3%), C_{16} (9%), C_{18} (2%) and C_{20} ($\leq$0.1%) and the C_{13}-C_{21} odd acids (total $\sim$ 1%).

b) Monoene acids

Monoenoic acids ranging from 14:1 to 24:1 make up 40-60% of the total fatty acids. The major members are listed below but many other acids are present at low levels.

palmitoleic acid	16:1, Δ9	10%
oleic acid	18:1, Δ9	16%
gadoleic acid	20:1, Δ11	10%
cetoleic acid	22:1, Δ11	7%
erucic acid	22:1, Δ13	

c) Polyene acids

The polyene acids in cod liver oil total some 18-30% of the component acids. Some of this variation arises from experimental error because of the difficulty of determining accurately acids of high unsaturation and molecular weights by gas chromatography[35]. The polyene acids range in chain length from C_{16} to C_{24} but the most common ones are those of C_{18}, C_{20} and C_{22} chain lengths belonging to the n-6 and n-3 series. Of these 20:5 (5, 8, 11, 14, 17) and 22:6 (4, 7, 10, 13, 16, 19) are usually the major components. Some of the more common acids of the n-3 and n-6

series are listed below.

n-6 acids

Trivial names	Shorthand notation	% composition of total fatty acids
Linoleic acid	18:2 (9,12)	1.8
-linolenic acid	18:3 (6,9,12)	0.1
-	20:3 (8,11,14)	0.1
Arachidonic acid	20:4 (5,8,11,14)	0.3
-	22:4 (7,10,13,16)	0.1
-	22:5 (4,7,10,13,16)	0.2
		Total = 2.6

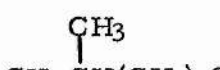
n-3 acids

α-linolenic acid	18:3 (9,12,15)	0.8
	18:4 (6,9,12,15)	1.5
	20:4 (8,11,14,17)	0.4
	20:5 (5,8,11,14,17)	9.2
	22:6 (4,7,10,13,16,19)	10.5
		Total = 24.3

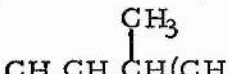
n-3/n-6 ratio = 9.3

d) Other acids

In addition to these major groups of acids, cod liver oil contains some minor components. These include a number of iso acids (C_{14}, C_{16}, C_{18}) which make up 1.4-1.6% and anteiso acids (C_{15}, C_{17}) which comprise ~0.3-0.5%. The structures of these two groups of acids are shown overleaf.



iso acids



anteiso acids

Also present are the isoprenoid acids (see section 2.2, page 34) and several furanoid acids (see section 2.3, page 50.)

1.2.2 Use of cod liver oil

The cod, Gadus morhua, and the herring, Clupea harengus, are among the greatest weight of fish landed in the world. The annual production of fish oils however fluctuates because of the varying catch of fish. European production of fish liver oils is on the decline but the major part of the production is cod liver and halibut liver oils.

Cod liver oil was produced and sold mainly for its content of the fat-soluble vitamins A and D. Veterinary cod liver oil has almost been replaced by synthetic vitamin products. Oils such as cod liver oil are, however, now attracting new interest on account of their content of polyunsaturated fatty acids believed to be important in human nutrition. In this context, it is utilised as a source of essential fatty acids. Essential fatty acids are those acids required for the maintenance of normal healthy growth, reproduction, and skin permeability. They are characterised by long chains with cis double bonds in methylene interrupted arrangement with the first double bond in position 6 from the methyl end of the molecule (the linoleic acid family). Of the three different families of polyene acids, the α-linolenic acid

and/or polyunsaturated acids of the n-3 family are essential fatty acids in fish[36].

Cod liver oil maintains dermal integrity and its essential fatty acid content ensures the proper utilisation of saturated fat. The highly unsaturated acids play a major role in the lowering of cholesterol levels, provide energy, and form an integral part of biomembranes. They are a substrate for the production of hormone-like substances, notably the prostaglandins.

Cod liver oil is used in animal feeds, mainly as fish meals, because of its growth-promoting effects, and as a cheap source of energy. It is among the marine oils used for industrial purposes such as protective coatings and for the manufacture of fatty acids. The production of margarines from the hydrogenated oil is an important industrial aspect of the fish oil industry.

In Britain today, fish body oil production is of little importance while fish liver oil industry has dwindled, but is still active. Significant amounts of cod liver oil are produced only at Hull (45%), Grimsby (35%), Fleetwood (8%) and Aberdeen (12%). The trend is towards still lower production affected adversely by the recent European Economic Community (EEC) policy on the fishing industry.

1.2 FATTY ACIDS IN COD LIVER OIL

1.2.1 Fatty acid structures

a) Saturated acids

The n-alkanoic acids of cod liver oil constitute 14-16% of the total acids[17]. These include the even acids: C_{12} (0.1%), C_{14} (3%), C_{16} (9%), C_{18} (2%) and C_{20} ($\leq$0.1%) and the C_{13}-C_{21} odd acids (total $\sim$ 1%).

b) Monoene acids

Monoenoic acids ranging from 14:1 to 24:1 make up 40-60% of the total fatty acids. The major members are listed below but many other acids are present at low levels.

palmitoleic acid	16:1, Δ9	10%
oleic acid	18:1, Δ9	16%
gadoleic acid	20:1, Δ11	10%
cetoleic acid	22:1, Δ11	7%
erucic acid	22:1, Δ13	

c) Polyene acids

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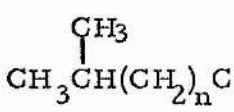
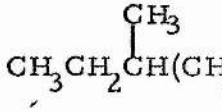


iso acids



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1.3 THE SEPARATION AND ISOLATION OF INDIVIDUAL FATTY ACIDS

1.3.1 Introduction

Fatty acid mixtures derived from fish oils differ from those obtained from other sources in being more complex. The acids differ in chain-length, degree of unsaturation, and double bond position. The isolation of an individual component is thus more difficult.

1.3.2 Distillation

Fractional distillation at low pressure permits separation mainly on the basis of chain-length. Methyl esters are preferred to fatty acids for distillation because they have lower boiling points. Under the conditions normally employed, polyunsaturated fatty acids, (especially those with three or more double bonds) may undergo double bond migration, stereomutation, and cyclisation. These problems are reduced in 'molecular distillation', but separation is then less efficient.

1.3.3 Low-temperature crystallisation

Crystallisation of acids, usually from acetone solution, at temperatures between 0° and -70° is a useful way of concentrating saturated monoene, and the various types of polyene acids. Separation is not complete but useful fractionations can be achieved and the method can be applied on a wide range of scale. The procedure is also a very mild one which can be safely applied to the labile polyenoic acids. The procedure has been reviewed by several workers[37-39].

1.3.4 Formation of urea inclusion compounds

The finding by Bengen that urea forms well-defined crystalline inclusion compounds with straight-chain compounds, but not with branched-chain or cyclic compounds, has been exploited in the isolation of individual fatty acids from natural mixtures. Urea forms inclusion compounds (adducts or complexes) with fatty acids or esters in which the straight-chain acids are held by secondary valence forces and by hydrogen bonds.

Urea formation has been used in the study of fish fatty acids or esters in two ways. Using a high proportion of urea to acid, almost all the straight-chain acids form an adduct leaving cyclic and branched-chain acids in the mother liquor along with some of the most highly unsaturated polyene acids.

With lower proportions of urea to acid, the natural mixtures can be separated into fractions in which saturated acids, monoene acids, and the several classes of polyene acids can be separated and concentrated. The application of urea inclusion compounds in the fractionation of complex fatty acid mixtures has been reviewed[40-42].

1.3.5 Adsorption chromatography

In adsorption chromatography solute molecules compete and are attracted to the active sites of the adsorbents by their polarity. Fatty acids with hydroxyl substituents can undergo hydrogen bond formation with the adsorbents. Conversely, exposed hydroxyl groups of the adsorbents can form hydrogen bonds with fatty acid molecules, especially if silicic acid is used as adsorbent.

a) Column chromatography

Separation of saturated or unsaturated acids/esters from compounds with additional polar groups (hydroxy or epoxy) is readily accomplished by column chromatography on silica. The more difficult separation of acids/esters depending on the number of double bonds results in concentration rather than isolation of pure compounds. Separation by unsaturation is more effective on silver column chromatography. The topic has been estensively reviewed by Morris[43].

b) Thin layer chromatography (tlc)

In thin layer chromatography a very fine grade silica gel (usually silica gel G) is applied to the plate, so that even layers of a pre-determined thickness are obtained.

In the handling of highly unsaturated compounds care must be taken to minimise autoxidation by operating in a nitrogen atmosphere and/or by addition of antioxidants. After development, the separated components are made visible by destructive (eg. phosphomolybdic acid) or non-destructive (eg. 2',7'-dichloro-fluorescein) sprays.

The use of salts which interact with the compounds being separated, such as silver nitrate or sodium borate, is a valuable extension to adsorption chromatography. Fractionation on tlc is sharper than on columns because the increased capacity per unit weight of adsorbent and because the larger ratio of adsorbent to sample size permits excellent separation.

c) High pressure liquid chromatography (HPLC)

HPLC at a pressure up to 500 psi, combines high speed, high efficiency, and high sensitivity. The application of HPLC to fatty acid analysis has been slow due principally to the problems of the detector systems. Of all the detectors (uv, heat of absorption, refractive index, fluorescence, flame ionisation, and polarography) refractive index detectors are more widely applied than others, though they are difficult to use with gradient elution. Uv detectors are very sensitive but fatty acids do not absorb uv light at the wavelength (254 nm) mostly used in HPLC instruments. Derivatisation of fatty acids into uv sensitive compounds is, however, possible.

Analysis of free fatty acids by HPLC using a fatty acid analysis-column and a differential refractometer detector has recently been developed by Waters Associates. A universal detector based on hydrogen flame is now available. Its potential in the analysis of fatty acids, including PUFA, has been demonstrated[44]. Non-volatile and or labile compounds can be handled without destruction, a feat impossible with the glc for example. The efficiency in HPLC (~5000 plates/m) is greater than that of glc (~2000 plates/m) and results obtained by HPLC are as accurate as those of glc. In future HPLC will compete with glc.

1.3.6 Partition chromatography

The separation of components by percolating a solution through a liquid phase is termed partition chromatography. Separation is based on differences in the partition coefficient of various solutes between two immiscible liquids.

a) Reversed-phase chromatography

In this system of chromatography, long-chain methyl esters are separated according to both chain-length and degree of unsaturation. The stationary phase is usually non-polar while the moving phase is a polar solvent. Reversed-phase chromatography is used for separation of lipids since these are retained more strongly by the non-polar stationary phase. The technique has been applied in both the column and tlc systems. Compounds that form 'critical pairs' in reversed-phase system can usually be separated using other methods.

b) Gas liquid chromatography (preparative glc)

The high separating power of glc on an analytical scale has been exploited at a preparative level for the isolation of pure fatty acids. Using preparative glc, submilligram and milligram quantities can be obtained on analytical size columns while gram quantities of material can be obtained on larger columns.

The commonest laboratory procedure for a small-scale preparative glc is to overload an analytical column with several milligrams of sample and to collect individual fractions through a sample collecting system at the column outlet. To be used on a small preparative scale the instrument must have the following features.

(i) An efficient flash heater for instant volatilisation of material and subsequent entry into the column as a narrow band.

(ii) A stream splitter at the end of the column, so that only a small fraction passes through the detector and the remainder is available for collection.

(iii) The outlet from the stream splitter to a collecting system should be heated to prevent condensation of non-volatile compounds which would lead to contamination.

There remain certain difficulties. Methyl esters issuing from the columns form aerosols that are not easily condensed and losses may be high. There is also a possibility that methyl esters of PUFA may be altered during preparative glc, especially at high temperatures. There are reports that esters such as 20:5 and 22:6 undergo isomerisation during preparative glc. This fact is relevant to the study of fish oils which frequently contain high proportions of such acids.

1.4 STRUCTURAL IDENTIFICATION OF NATURAL FATTY ACIDS

In the elucidation of structure of fatty acids, chemical and physical procedures are used, though presently the latter is more widely used. Physical methods, such as mass spectrometry and nmr are now indispensible tools of analysis though chemical methods such as hydrogenation and ozonolysis are still popular and widely used.

One major problem in fatty acid structural identification, is that no one method (physical or chemical) is capable of indicating the chain-length of an acid, the extent of unsaturation, the relative positions of the double bonds, cis and trans isomers, and the presence and nature of functional groups including type and extent of branching.

1.4.1 Hydrogenation

Hydrogenation, in conjunction with the glc identification of perhydro compounds on polar and non-polar columns, is very useful in the assignment of unidentified peaks. The changed chromatographic behaviour after hydrogenation may give a clue to the type of acid originally present. Hydrogenation does not however affect n-saturated fatty acids, saturated branched-chain or cyclic acids, nor does it affect saturated hydroxy acids.

1.4.2 Double-bond positions by oxidative cleavage

a) Periodate-permanganate

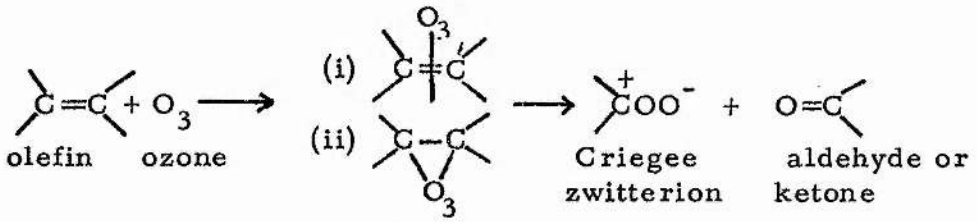
At the concentration of potassium permanganate and sodium metaperiodate used (in a molar ratio of 1:39), potassium permanganate is quickly regenerated from manganate. The first stage of oxidation of unsaturated acid by permanganate is the production of a 1,2-diol (pH 8-9) and α-ketol (pH 7-8). The end products are mono- and

dicarboxylic acids from monoenes along with malonic acid from methylene-interrupted polyenes. The method is generally suited and applicable to monoenes; it is less satisfactory with polyenes.

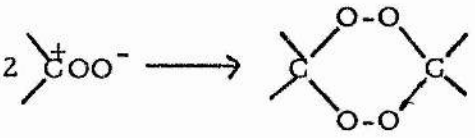
b) Ozonolysis

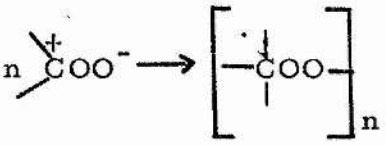
The reaction of ozone with double bonds and cleavage of the resulting products followed by their analysis and identification is the most widely employed method of double bond location. Ozone attacks unsaturated fatty acids rapidly and quantitatively. The mechanism and formation of ozonides has recently been reviewed by Criegee[45] and Pryde[46].

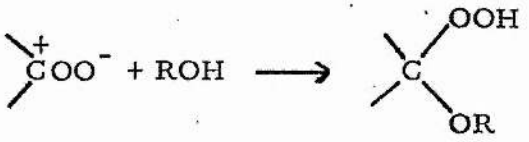
Ozone reacts with double bonds to give an unstable primary product which quickly transforms through an intermediate into a Criegee zwitterion and an aldehyde or ketone. The zwitterion then reacts with a protic solvent if present to form alkoxy or acyloxy hydroperoxide, or reacts with aldehyde or ketone to give an ozonide and/or polymeric ozonides. Alternatively, the zwitterion can form a polymeric peroxide or undergo a rearrangement process as shown below:-

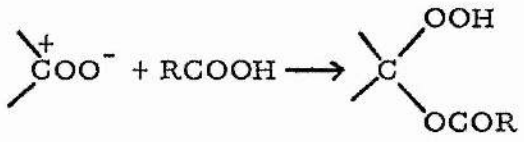


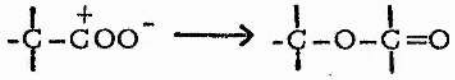
(a) Ozonides:

(b) Dimer Peroxides: 

(c) Polymeric Peroxides: 

(d) Alkoxy hydroperoxides: 

(e) Acyloxy hydroperoxides: 

(f) Rearrangement: 

With participating solvents (water, alcohols, organic acids etc.) hydroperoxides predominate, but with non-participating solvents (paraffin, $CHCl_3$, CCl_4, acetone etc.), the principal product is the monomeric ozonide.

The main factors which affect and control the formation of ozonides include the nature of reacting olefin, the temperature of ozonisation, the solvent, and the technique of ozonisation employed. Perhaps solvent effect is the most important factor which determines and controls ozonolysis. Examples of use of different solvents abound in the literature and recent reports indicate that ozonisation of 1, 2-disubstituted ethylenes in an alcohol medium in the presence of anhydrous HCl results in the formation of ester fragments[47].

Reductive cleavage of ozonolysis products is the preferred method of cleavage. Reduction of ozonides yielding aldehyde and

aldehydo-esters has successfully been carried out using a number of reagents. Triphenylphosphine (TPP) has been recommended by several workers[48-52]. The reduction of ozonides to alcohols and alcohol esters with sodium borohydride ($NaBH_4$) as developed by Sousa et al[53], is also used for the double bond location of diene fatty acids[54].

1.4.3 Double-bond position and configuration by spectroscopic procedures

a) Ultra-violet Spectroscopy (uv)

Uv spectroscopy was one of the earliest physical techniques to be applied to the identification and characterisation of PUFA. It is particularly useful for the detection of conjugated polyenes which absorb uv light at specific wavelengths. Alkali treatment of polyenes having double bonds separated by a single methylene group gives conjugated unsaturation; subsequent analysis of the resulting mixtures by uv spectroscopy will indicate the presence of conjugation. The uv spectroscopic method has been a classical technique for the detection of methylene-interrupted systems. However, since the advent of glc application of alkaline isomerisation and uv spectroscopy for the identification of polyunsaturated fatty acids is mainly qualitative.

b) Infra-red spectroscopy (ir)

In fatty acid structural elucidation ir spectroscopy is particularly useful for the detection of trans double bonds. The application of ir spectroscopy in the study of fatty acids has been reviewed by O'Connor[55].

c) Nuclear magnetic resonance (nmr)

Nmr spectroscopy is a powerful tool for the structural elucidation of fatty acids and their derivatives. This followed marked improvement in the use of proton magnetic resonance (PMR) and ^{13}C carbon magnetic resonance (^{13}CMR) for the characterisation of fatty acids. The introduction of super-conducting magnets, Fourier transformation, coupled with pulse techniques and use of chemical shift reagents has further improved the technique. The subject of nmr and its application to lipid analysis has been reviewed by several workers[56-59].

d) Mass spectrometry (MS)

(i) Double-bond derivatives

The chemical modification of the unsaturated centres of fatty acids and analysis of the resultant derivatives by mass spectrometry has been applied to the location of double bonds of various acids by gas chromatography-mass spectrometry (GC-MS). Particularly valuable is the analysis of the trimethylsilyl (TMS) derivatives following complete hydroxylation of the double bonds[61]. A microprocedure for double bond location in polyene acids through oxymercuration and GC-MS has also been developed[62].

(ii) Pyrrolidides

Pyrrolidides of polyene acids, unlike the methyl esters, give different spectra for isomeric acids. Consequently both the position of double bonds and methyl branching in the fatty acids can be determined with accuracy. This technique first used by Andersson et al[63] for monoene acids and later extended for polyenes[64], has been recently reviewed[65].

RESULTS AND DISCUSSION

2.1 STRAIGHT-CHAIN SATURATED FATTY ACIDS

2.1.1 Introduction

The occurrence and distribution of fatty acids of fish oils has been investigated by several workers[66-69]. The results show that the composition of the fatty acids of fish oils are controlled by environmental factors, especially the nature of the diet[70-74] and by the water temperature.

It has been shown[75-77] that as the temperature of the water falls, there is an increase in the proportion of C_{20} and C_{22} fatty acids compared to the C_{16} and C_{18} acids and an increase in the degree of unsaturation. This represents an attempt by the fish to maintain cell lipids in a fluid state at reduced temperature. In addition, fish have control mechanisms which determine the degree of unsaturation of fatty acids when the temperature of the water changes. Temperature variation also affects the fatty acid composition of the dietary lipids. Hence the fatty acid composition of cultured herring "reflects the dietary fatty acid pattern, except for the C_{16} and C_{18} saturated and monoenoic fatty acids, where some interconversion or preferential oxidation seems to have occurred"[78].

The saturated fatty acids in fish constitute about 25% of the total acids. The major acid - palmitic (15-20%) - is accompanied by myristic and stearic acids[6]. The predominance of palmitic acid among the saturated fish acids may be explained by the following factors. (a) Palmitic acid is derived from diets lower down the food chain which have this acid as the major saturated fatty acid. Ackman et al, in a comparative study of fatty acids of different groups of phytoplankton[79] and of some marine seaweeds[80], pointed

to the dominance of palmitic acid amongst other saturated acids. (b) The presence of a significant amount of palmitic acid in fish may arise from de novo synthesis. Palmitic acid is the main end-product of fatty acid synthetase in both mammals and fish[81] and fatty acids of chain lengths longer than C_{16} are subsequently synthesised by further stepwise addition of two-carbon units. Thus oleic acid is derived from palmitic acid by elongation and $\Delta 9$ desaturation[82].

The chain length of the straight-chain saturated fatty acids of fish range from C_{12} to C_{22}. The major acids are the C_{14}, C_{16} and the C_{18} acids and the minor acids include the C_{13}-C_{21} odd carbon members and some even carbon chain acids (C_{12}, C_{20}, C_{22}). A complete and satisfactory estimate of all these acids in a one-step analysis is not practicable. A problem in superimposition by unsaturated acids and branched-chain compounds, especially on polar columns would be experienced with glc. Moreover, a complete and satisfactory isolation of the straight-chain acids from branched-chain ones has always been a daunting task for lipid analysts.

Several isolation procedures have been applied to this problem. Low temperature crystallisation has been used for the crude separation of saturated and unsaturated acids[41]. Ackman and Sipos have used silver nitrate-silicic acid chromatography for the isolation of saturated acids from fish oils[83]. Perhaps the most widely used separation technique for the isolation of saturated from branched-chain acids is urea fractionation. Cason et al[84] separated branched-chain from n-fatty acids on columns of

urea:celite (5:1 w/w) eluting the column with methanol saturated with urea. The preferred method of isolation of straight-chain acids is crystallisation from a suitable solvent in the presence of urea. By this means higher straight-chain fatty acids (C_{18} and above) are conveniently separated as urea adducts. Shorter chain length n-fatty acids do not however form urea adducts so completely and are more difficult to isolate by this technique. Countercurrent distribution using acetonitrile-hexane is another good technique and has proved useful in industry for the isolation and enrichment of n-fatty acids. The potential of the countercurrent distribution procedure has been outlined by Dutton[85].

Separation of n-saturated fatty acids from polyunsaturated members has also been achieved using both reversed-phase chromatography[86-88] and high pressure reversed-phase liquid chromatography. Pei et al[89] used a chemically bonded reverse phase packing (VYDAC reversed-phase column) to separate fatty acids according to chain length and degree of unsaturation

2.1.2 Isolation of n-saturated esters

In this study urea treatment was used for the isolation of the straight-chain fatty acids. Free fatty acids were used in preference to their methyl esters because urea binds polyenoic esters easier than the acids and because dissolution of methyl esters in most solvents employed for urea fractionaton (e.g. methanol) is sometimes incomplete. Free fatty acids (50 g) and urea (50 g) were heated in methanol until solution was complete. The solution was cooled at room temperature and kept at 0^{o} overnight. The

detailed procedure is given in the experimental section (page 127). The glc analysis of the total fatty acids, the mother liquor, and the adduct (all as methyl esters) is presented in Table 1 (page 28).

Saturated fatty acids (16% in the total ester), concentrated in adduct 1 (37%), were accompanied by monoene acids (62%). A second treatment of the adduct with urea gave adduct 2 with 61% of saturated acids and 36% of monoene acids (see Scheme 1). This second adduct was then converted to methyl esters and separated into two major fractions by thin layer silver ion chromatography. The more mobile band was mainly saturated esters (98%) and the less mobile band was mainly monoene esters (89%).

Preparative gas liquid chromatography (glc)

Isolation of individual fatty acids was finally achieved using preparative glc on 20% EGSS-Y. Details of the preparative glc procedure are as previously described except that the outlet temperature was set at 260°. Each fatty acid was concentrated under a gentle stream of nitrogen and the weight noted. Purity was checked by glc (10% SP 2340), and the C_{14}, C_{16}, C_{18} and C_{20} ester fractions obtained were in excess of 99% purity.

2.1.3 Structural identification

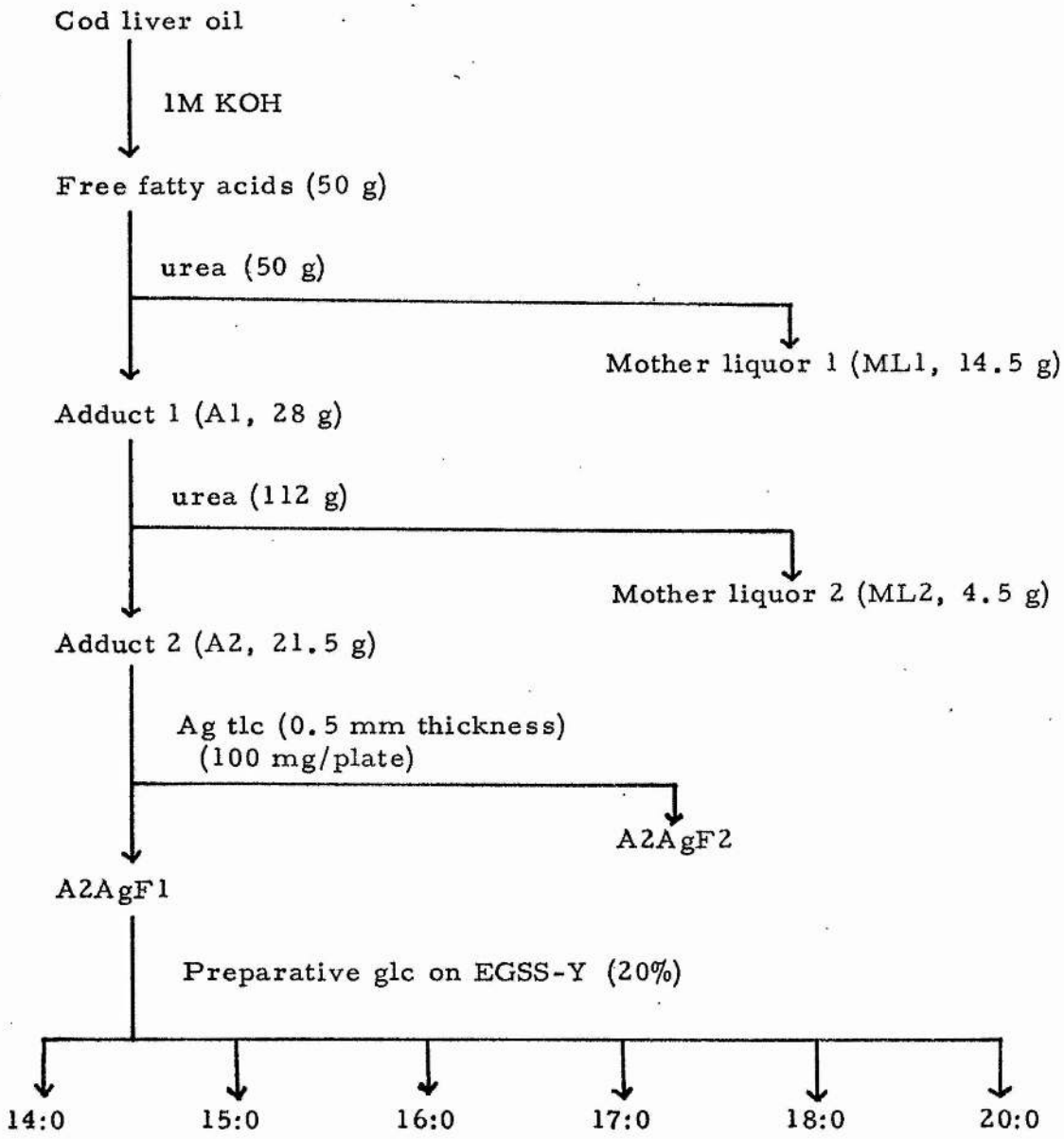
Identification of the isolated components of the straight-chain saturated fatty acids was also undertaken using a combination of mass spectrometry and glc (see pages 30 and 32).

Table 1. Component acids (% wt, SP-2340 columns) of total lipids, adduct 1 (A1), adduct 2 (A2), adduct 2-silver ion separated fraction 1 (A2AgF1) and fraction 2 (A2AgF2), mother liquor 1 (ML1) and mother liquor 2 (ML2)

Assignment	Total	A1 [66%]	A2 [54%]	A2AgF1	A2AgF1 (3% SE30)	A2AgF2	ML1 [34%]	ML2 [12%]
14:0	3.4	5.4	3.8	6.0	6.5	0.6	2.1	5.6
15:0	0.3	0.6	1.2	1.2	0.7	-	-	0.6
16:0	10.0	24.8	39.4	62.8	65.8	2.6	1.2	17.7
16:1	9.1	3.4	-	-	-	1.5	10.8	4.5
18:0	2.5	6.1	15.4	27.5	24.1	0.7	0.9	2.0
18:1	21.9	19.6	6.6	-	0.8	15.4	21.0	23.9
18:2(n-6)	1.9	0.3	0.2	-	-	-	3.0	0.6
20:1/18:3(n-3)	14.6	19.7	10.9	-	-	26.1	10.6	23.7
18:4(n-3)	2.4	-	-	-	-	-	4.1	0.2
22:1/20:4(n-6	8.8	18.8	18.6	-	-	45.6	2.7	19.8
20:4(n-3)	0.7	-	-	-	-	-	1.2	-
20:5(n-3)	10.8	0.2	-	-	-	-	17.5	0.2
22:4(n-6)	0.9	1.3	2.4	-	-	-	0.5	-
22:5(n-3)	0.8	-	-	-	-	-	1.7	-
22:6(n-3)	11.8	-	-	-	-	5.9	19.6	1.1
Others	0.1	-	1.3	2.3	2.1	1.7	1.2	-

Note: [%] - indicates percentage of material in each of these fractions

Scheme I Flow diagram for the isolation of the straight-chain saturated fatty acids from cod liver oil



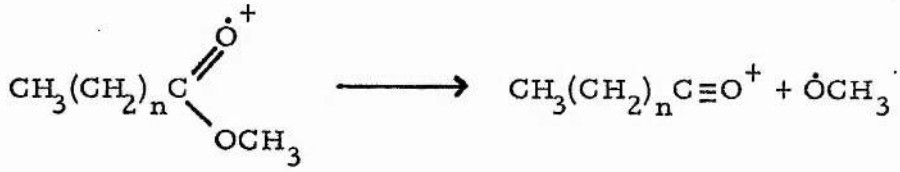
(a) Identification by mass spectrometry

(i) Authentic methyl hexadecanoate (16:0)

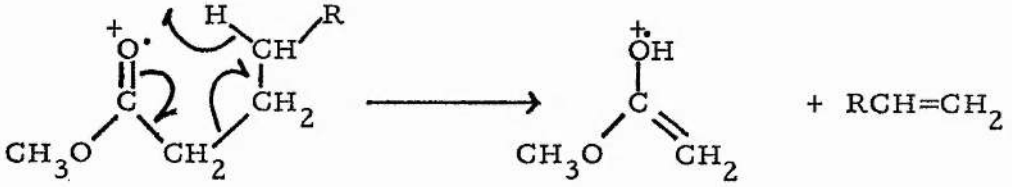
The mass spectrum of methyl hexadecanoate (page 142) was examined to assist in the interpretation of the spectra obtained from the esters isolated from cod liver oil.

The spectrum shows a molecular ion peak at m/e 270 (1.4%) and fragment ions at m/e 241 (0.7%), 239 (M-31, 1.4%), 227 (2.4%), 213 (0.7%), 199 (1.0%), 185 (1.4%), 171 (1.4%), 157 (0.7%), 143 (7.6%), 129 (3.8%), 115 (1.7%), 101 (3.5%), 87 (62.1%) and 74 (100%).

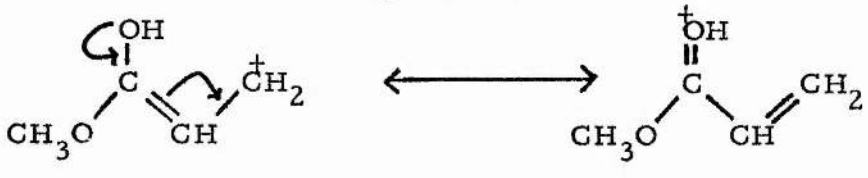
The acylium ion at m/e 239 (M-31) results from cleavage of the ester function thus:



The base peak at m/e 74 results from McLafferty rearrangement and is common to all methyl esters which have an H atom on C-4 and are not substituted at C-2. It arises from the following fragmentation:



The remaining ions listed above belong to the series $[(CH_2)_nCOOCH_3]^+$ of which that of m/e 87 (n=2) is the most abundant. This derives its stability from resonance:



Dinh-Ngugen et al[90], from a study of ^2H-labelled compounds, conclude: (a) there is extensive hydrogen interchange along the chain,

(b) whilst the ions of mass m/e 87, 101, 115, and 129 arise from systematic cleavage of the chain the fragments of higher m/e result through expulsion of intermediate portions of the chain. For example, the M-29 ion arises almost exclusively by loss of the C-2 and C-3 methylene group.

(ii) Methyl tetradecanoate (14:0)

The mass spectrum of this ester (page 142) shows a molecular ion at m/e 242 (3.5%), the acylium ion at m/e 211 (2.2%), and a base peak at m/e 74. Ions of the series $[(CH_2)_nCOOCH_3]^+$ are apparent at m/e 241 (0.1%), 227 (0.1%), 213 (2.2%), 199 (3.0%), 185 (0.9%), 171 (0.4%), 157 (0.9%), 143 (6.1%), 129 (2.6%), 115 (0.1%), 101 (3.5%) and 87 (56.6%).

(iii) Methyl hexadecanoate (16:0)

The mass spectrum of this ester (page 143) displayed a similar fragmentation pattern to the authentic methyl hexadecanoate already discussed. The molecular ion was observed at m/e 270 (3.5%), the acylium ion at m/e 239 (1.7%) and a base peak at m/e 74. Fragment ions of the series $[(CH_2)_nCOOCH_3]^+$ were observed at m/e 241 (0.7%), 227 (2.6%), 213 (0.3%), 199 (1.3%), 185 (1.7%), 171 (1.3%), 157 (0.9%), 143 (7.0%), 129 (3.5%), 115 (1.3%), 101 (3.9%), and 87 (56.6%).

(iv) Methyl octadecanoate (18:0)

The mass spectrum of this ester (page 143) showed a molecular ion at m/e 298 (4.3%), which is evidence of a saturated structure. The acylium ion at m/e 267 (1.4%) confirmed the validity of the molecular ion and the base peak occurred at m/e 74. The

fragmentation patterns are similar to those already discussed. Ions of the series $[(CH_2)_nCOOCH_3]^+$ were observed at m/e 269 (0.4%), 255 (2.6%), 241 (0.4%), 227 (0.4%), 213 (1.1%), 199 (2.1%), 185 (1.4%), 157 (0.1%), 143 (9.3%), 129 (3.2%), 115 (1.4%), 101 (3.6%) and 87 (53.6%).

(v) Methyl eicosadecanoate (20:0)

The mass spectrum of this ester did not show the expected molecular ion at m/e 326. Although the purity of this ester was > 99%, the amount was very small (0.02 mg). The base peak at m/e 74 due to McLafferty rearrangement ion was present and confirmed the component as a methyl ester. The second largest peak occurred at m/e 87 and other series of ions as already discussed with other methyl esters were present. The mass spectrum of this component therefore failed to prove the structure of the C_{20} fatty acid. Tentative identification of this ester must therefore rest on chromatographic behaviour.

(b) Identification by glc

Both polar and non-polar columns were used for the analysis and tentative identification of the isolated acids. Equivalent chain lengths were calculated from a standard reference line obtained by plotting the logarithms of the retention times of C_{14}-C_{24} normal saturated monocarboxylic methyl esters against the number of carbon atoms in the acid. The ECL values obtained from 10% SP-2340 (polar), 3% SE-30, and 20% EGSS-Y columns showed these acids to be saturated.

Conclusions regarding the structures of these saturated acids are therefore based on the following observations.

(a) The acids were obtained from adducts after repeated urea fractionation. As already pointed out only straight-chain acids form urea adducts. These are mainly saturated but contain also some monoene compounds.

(b) The straight-chain saturated acids were conveniently separated from the monoenes by argentation chromatography (tlc). With PE5 as developing solvent saturated esters migrate well ahead of monoenes and are readily separated from them.

(c) In gas liquid chromatography on several phases, they always behave like authentic saturated esters.

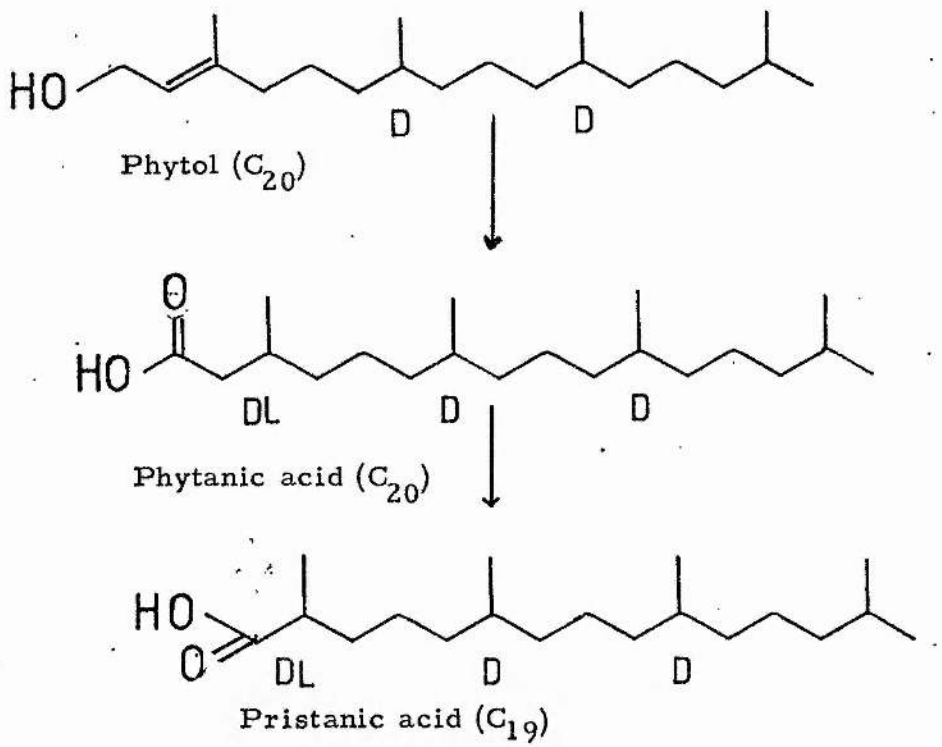
(d) The mass spectra of the various isolated esters finally confirmed their structure. The molecular ion confirmed the molecular weight and other characteristic ions showed these acids to be saturated and straight-chain.

2.2 ISOPRENOID ACIDS

2.2.1 Introduction

The origin and widespread occurrence of isoprenoid acids in fish oils has been traced to the food sources of these organisms within which an efficient but complex predator-prey interrelationship has evolved into a marine food web[91]. A direct food chain link has been established between phytoplankton and the Antarctic whales[92]. The antarctic krill (Euphansia supberba Dan) feed on the phytoplankton and eventually are eaten by the Antarctic whales.

Terrestial mammals and microorganisms have been shown to convert phytol (3,7,11,15-tetramethylhexadec-2-en-1-ol) into phytanic acid (3,7,11,15-tetramethylhexadecanoate) with smaller amounts of pristanic acid (4,8,12-trimethyltridecanoate) and traces of phytenic acid (3,7,11,15-tetramethylhexadec-2-enoic acid)[93-96] as shown below.

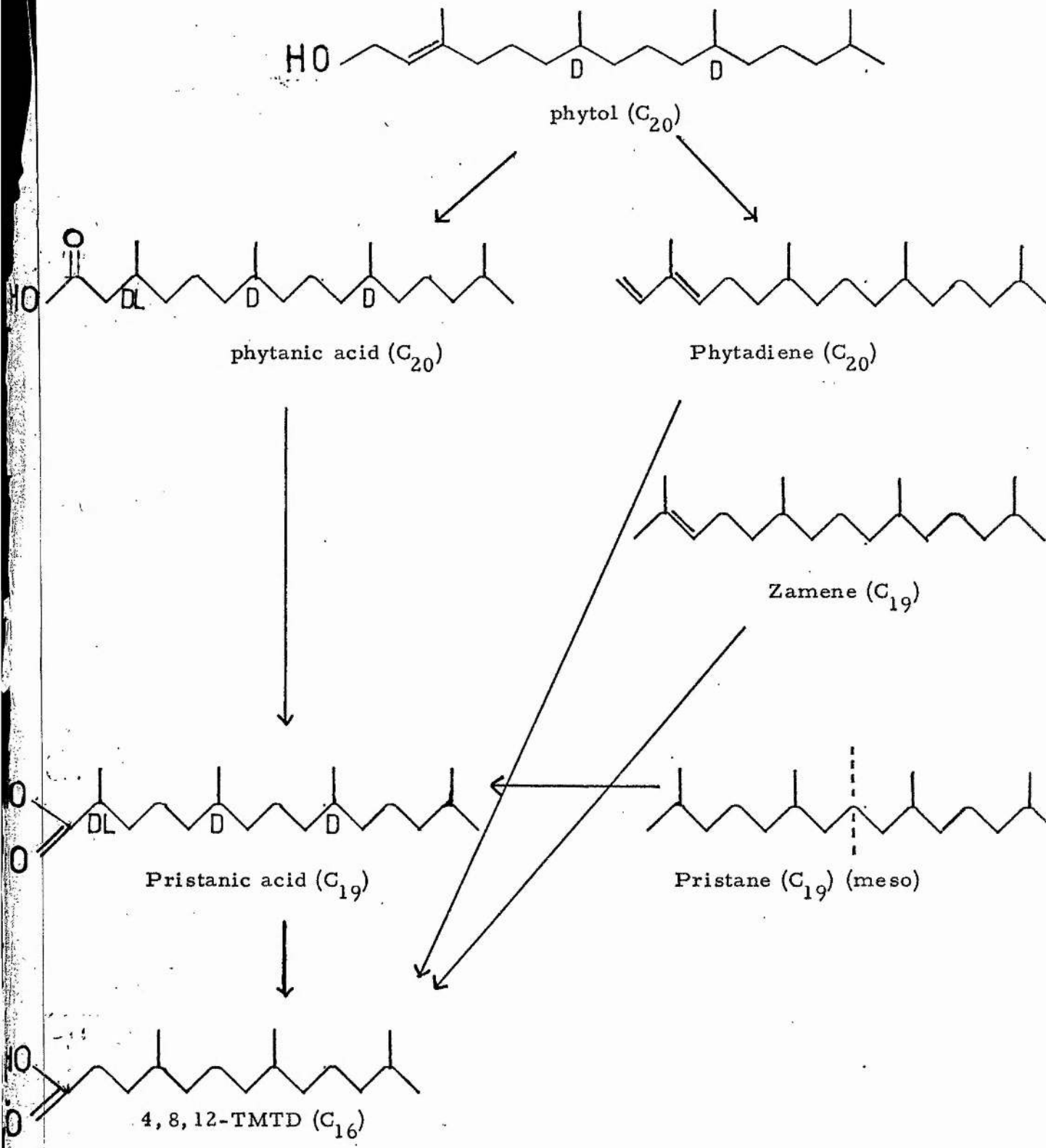


Endogenous synthesis of phytanic acid does not take place in either mammals or fish. Isoprenoid acids found in most fish are derived from seaweeds and the food web. The following general scheme (see Scheme II) is now offered as possible pathways to isoprenoid acids in fish.

In the aquatic environment unsaturated hydrocarbons, such as zamene and phytadiene, are derived from phytol by oxidation-decarboxylation and dehydration respectively. These unsaturated hydrocarbons form a major food source for zooplankton and when liberated into the ocean they undergo some oxidation at the double bond to form 4, 8, 12-trimethyltridecanoic acid. This acid later re-enters the aquatic food web via filter feeders. It has been suggested that pristane also is oxidised to pristanic acid[97]. Pristane may be derived from the precursor, phytol, with preservation of the original stereochemistry as the meso form; and the conversion of phytol to pristane may be responsible for its widespread occurrence in the aquatic food web[98]. Zooplankton have been shown to contain appreciable amounts of zamene (2, 6, 10, 14-tetramethylpentadec-2-ene) and phytadiene (3, 7, 11, 15-tetramethylpentadeca-1, 3-diene)[99] and since zooplankton are among the chief sources of food for most fish, the occurrence of isoprenoid acids is not surprising.

Various techniques have been used for the isolation of isoprenoid acids from different sources. Hansen and Shorland[100] employed fractional distillation, adsorption chromatography and low-temperature crystallisation, while Sonneveld et al[101] used fractional distillation, urea adduct-formation and countercurrent distribution, followed by hydrogenation and preparative gas liquid

Scheme II. Possible pathways for the exogenous synthesis of isoprenoid acids present in fish.



chromatography.

Cod liver oil contains an appreciable amount of C_{16} monoene acid which overlaps with the multi-branched C_{20} fatty acid (phytanate) on polar columns in gas chromatography. Moreover, the total concentration of these isoprenoid acids in cod liver oil is very small (probably 0.01 to 1.0%).[102] For these reasons, isolation of isoprenoid acids from most fish oils is not easy. The preferred method for the isolation of all branched-chain compounds involves urea fractionation[80-92,103]. The isoprenoid acids do not form urea complexes and thus remain in the mother liquor where they will be accompanied by some other branched-chain acids, furanoid acids, polyene acids, some of the shorter chain acids, and some non-lipid material.

2.2.2 Isolation

In the present investigation isoprenoid acids were isolated from the cod liver oil fatty acids by urea fractionation, silver ion chromatography and preparative gas liquid chromatography as indicated in Scheme III.

The methyl esters were first crystallised with urea and methanol using 2 g of urea per g of ester. The esters recovered from the adduct and from the mother liquor had the compositions summarised in Table 2. The esters which did not form an initial adduct were submitted to a second treatment with urea (5 g per g of ester) and these fractions had the approximate compositions shown in Table 2.

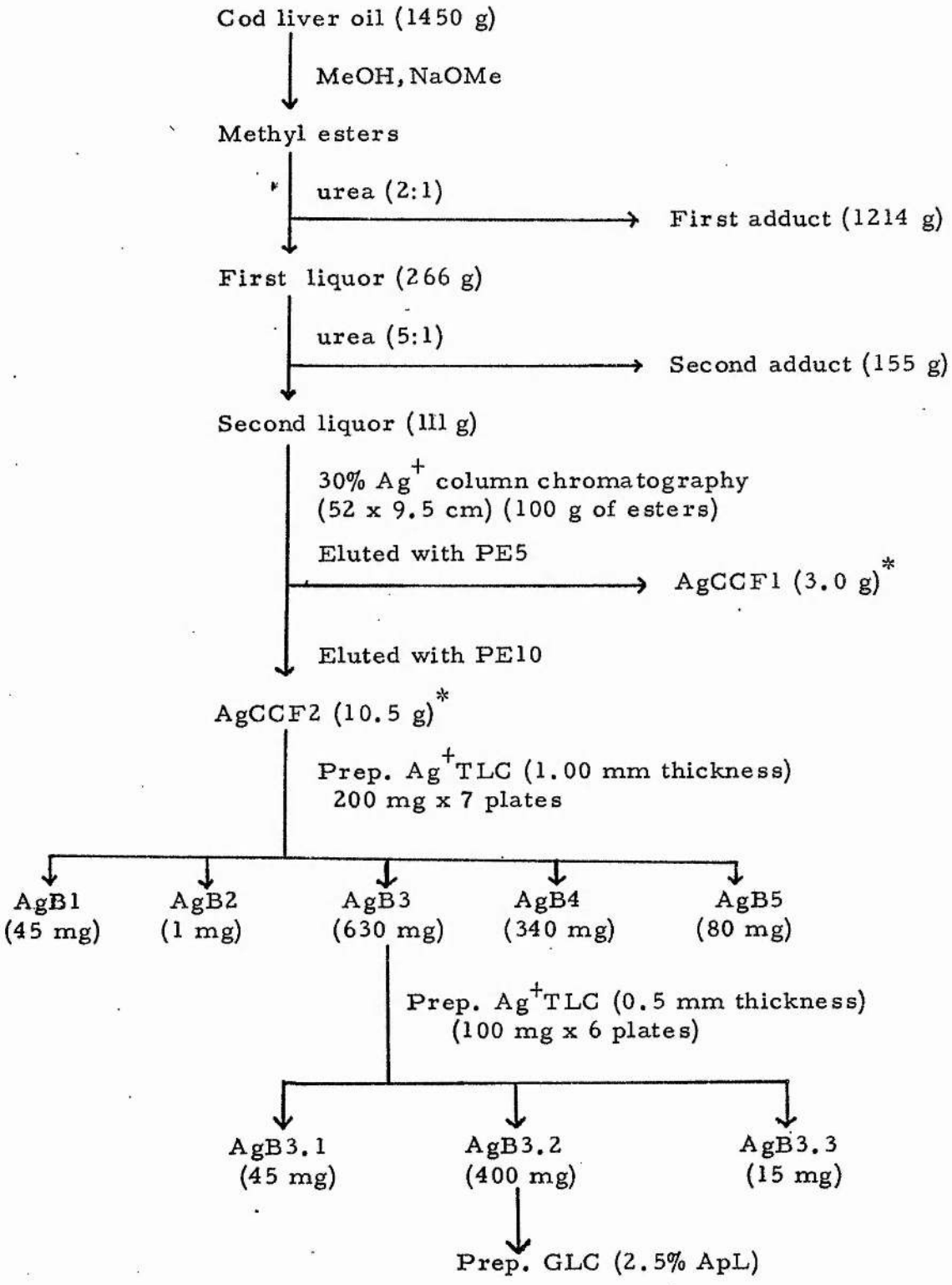
The second liquor was eluted from a column of silica containing silver nitrate with a range of solvents of increasing

polarity. GLC analysis showed that the polyenes had been largely retained on the column. Analytical tlc (both silver and ordinary silica tlc) was carried out on the eluted fractions AgCCF1 and AgCCF2 alongside authentic samples of phytanate, monoene, and saturated esters. AgCCF1 contained two bands of almost equal amounts. The faster moving band was presumably hydrocarbons, while the other band contained methyl esters.

Table 2. Component acids (% wt, SP2340 columns 185°) of total lipids, adduct 1 (A1), adduct 2 (A2) and mother liquor 2 (ML2).

Assignment	Total	A1	A2	ML2
14:0	3.4	3.8	0.1	1.9
15:0	0.3	0.5	0.2	0.1
16:0	10.0	18.5	0.1	-
16:1	9.1	7.0	1.3	4.5
18:0	2.5	3.7	-	-
18:1	21.9	28.6	2.4	1.4
18:2 (n-6)	1.9	0.1	2.4	2.0
20:1/18:3 (n-3)	14.6	16.1	1.9	-
18:4 (n-3)	2.4	0.4	6.7	7.4
22:1/20:4 (n-6)	8.8	7.8	1.5	0.9
20:4 (n-3)	0.7	-	2.4	-
20:5 (n-3)	10.8	3.8	32.5	15.1
22:4 (n-6)	0.9	1.7	1.7	1.1
22:5 (n-3)	0.8	1.4	4.4	0.4
22:6 (n-3)	11.8	4.0	36.5	58.9
Others	0.1	2.6	6.1	6.3

Scheme III. Flow diagram for isolation of isoprenoid acids



* 86.5 g Methyl esters were retained on the column

The two fractions (AgCCF1 and AgCCF2) were chromatographed separately on silica to free the methyl esters of non-ester contaminant. The composition of the two fractions (AgCCF1 and AgCCF2), together comprising about 1% of the total methyl esters, is detailed in Table 3.

Table 3. Composition (% wt) of AgCCF1, AgCCF2 and AgB3.2 (SP-2340 at 185°)

Tentative Assignment	AgCCF1	AgCCF2	AgB3.2
P_{16}	0.2	11.5	20.6
P_{17}	0.5	1.0	-
P_{19}	32.6	10.1	14.4
P_{20}	66.7	32.3	65.0
Other branched-chain esters	-	9.8	-
Furanoid esters	-	35.2	-

Fraction 1 appears to be mainly the P_{19} and P_{20} esters whilst fraction 2 also contains some P16, other branched-chain esters and some furanoid esters.

This result indicates that the isoprenoid acids are eluted from silver ion columns by PE5 in the order P_{20} before P_{19}, before P_{17}, before P_{16}. Further silver ion chromatography of AgCCF2 (see Scheme III) produced a fraction containing only isoprenoid acids.

Preparative glc (ApL, 3.5%) was then used to isolate each of the isoprenoid acids in pure form prior to structural determination by mass spectrometry. The isoprenoid band (AgB3.2) was separated into three distinct peaks corresponding to the C_{16}, C_{19} and C_{20}

compounds. The C_{17} acid , present in a trace amount was not isolated by this technique but the rest were trapped and recovered as detailed in the experimental section (page 126).

A split ratio of 100:1 in favour of the collector was used and recovery of esters was in excess of 60%. Sudden cooling of separated components on contact with chloroform produced aerosol formation with some loss of material and certain measures were adopted to reduce or eliminate this. In particular the temperature of the collecting vials was kept at 0-10°C and samples not exceeding 5-7 mg were applied to the column; at this level separation of various components was complete.

The degree and extent of contamination was affected by the temperature of the exit tube. A slight increase of temperature above column temperature helped to reduce contamination. Injection of distilled hexane between each peak also helped to improve the purity of isolated compounds[104]. The ECL and % purity of isolated components by preparative glc are detailed in Table 4.

Table 4. ECL and % purity of the P. acid ester components after preparative glc

Assignment	ECL ApL, 3.5%, 200°	ECL SP2340, 10%, 185°	Purity %
P_{16}	14.3	14.1	100.0
P_{19}	16.4	15.6	99.9
P_{20}	17.5	16.9	100.0

2.2.3 Biosynthetic speculation

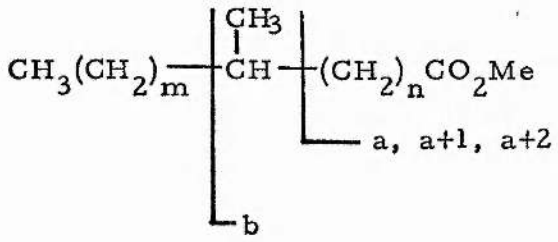
The three phytol-based esters (P_{16}, P_{19}, P_{20}) were identified by a combination of glc behaviour and mass spectrometry in

comparison with known information about these compounds.

a) General comments on the mass spectra of branched-chain acids

There is now available, a considerable amount of information about MS fragmentation of branched-chain esters which has been reviewed by McCloskey[60] and Abrahamsson et al[105] and discussed by Apon and Nicolaides[106] and Lough and Smith[107] among others.

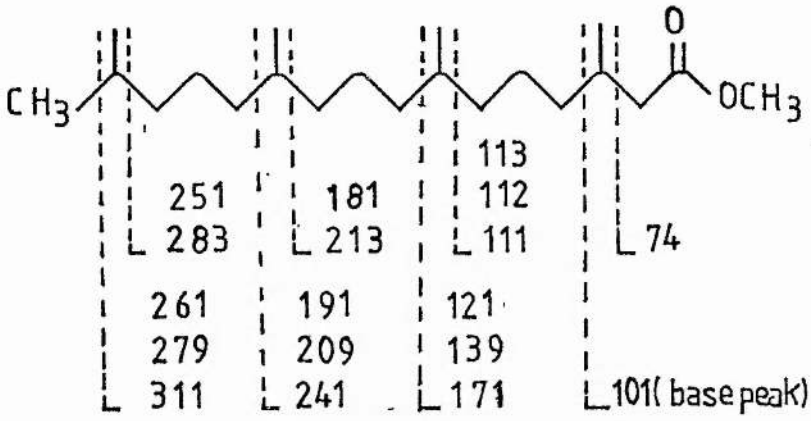
In general a branched-chain ester undergoes cleavage on



either side of the branched-chain carbon atom to give fragments a and b. With a methyl branch, these differ by 28 mass units and the peaks at a and b are much larger than the peak at a+14 which would be obtained in the absence of branching at that point. In addition, fragment a is accompanied by smaller peaks at a+1, and a+2, and fragments a+b may each lose methanol and water sequentially.

A different kind of fragmentation produces peaks at M-29 through loss of the C-2 and C-3 methylene groups and hydrogen, and at M-43 through loss of the C-2 to C-4 methylene groups and hydrogen. These figures will be modified if any of the methylene groups in these positions carry a branched methyl group. A significant peak is also expected at m/e 74 due to McLafferty rearrangement. This will have a higher m/e value if the ester is substituted at C-2.

b) Mass spectrum of the P_{20} methyl ester



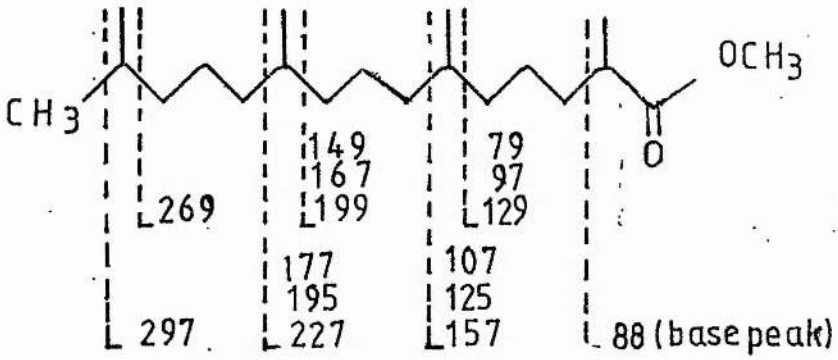
The P_{20} methyl ester isolated by preparative glc was submitted to mass spectrometry (page 144). A molecular ion at m/e 326 (13%) indicates the methyl ester of a C-20 acid and the presence of a significant peak at m/e 74 (65%) confirms the existence of a methyl ester and the absence of substitution at C-2.

The base peak at m/e 101 is a clear indication of a 3-methyl derivative. Other branched methyl groups at C-7 (m/e 143, 144, 112, 111 and 171), C-11 (m/e 213, 214, 215, 181 and 241) and C-15 (m/e 283, 285, 251 and 311) are indicated by the peaks listed in parenthesis. The first and last peak in each group result from cleavage on either side of the branched methyl group. The formation of other fragment ions has been discussed in section a).

There is good reason therefore to believe that the sample under investigation (P_{20}) is methyl 3,7,11,15-tetramethylhexadecanoate (phytanate). The fragmentation patterns observed here were similar to those reported elsewhere[105,108,109]. Similar results were observed with an authentic sample of methyl phytanate and in a GC-MS spectrum obtained from a mixture of the phytol-based

esters.

c) Mass spectrum of the P_{19} methyl ester



This ester is shown to be methyl pristanate (2, 6, 10, 14-tetramethylpentadecanoate) on the basis of the following characteristic peaks.

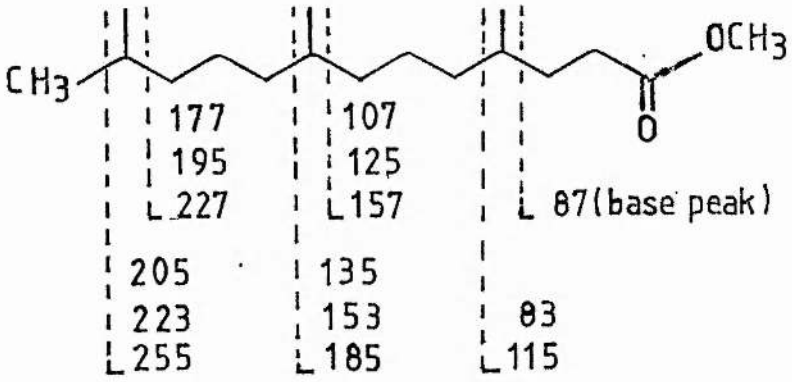
The molecular ion (m/e 312 (22%)) indicates the methyl ester of a saturated C-19 acid, and the base peak at m/e 88 (rather than the frequently encountered m/e 74) shows the presence of methyl branching at C-2. This is supported by a small but characteristic ion at m/e 253 (M-$COOCH_3$).

The presence of a second methyl group on C-6, follows from the appearance of peaks at m/e 129, 130 and 97, and 157, 125 and 107. Further evidence for this, is the presence of a peak at m/e 222 (M-90). Sen Gupta et al[110] and Meyersen and Leitch[111] have independently shown that the 6-methyl substituted acids produce a fragment ion of M-76 through loss of CH_3OH and CH_2=CHOH. This fragment will be at M-90 because of the methyl group on C-2. Jacob and Poltz[112] and Jacob[113] have previously reported that 6-methyl substituted esters show an intense M-76 ion, which is shifted to M-90 if an additional methyl branch is located in C-2 position.

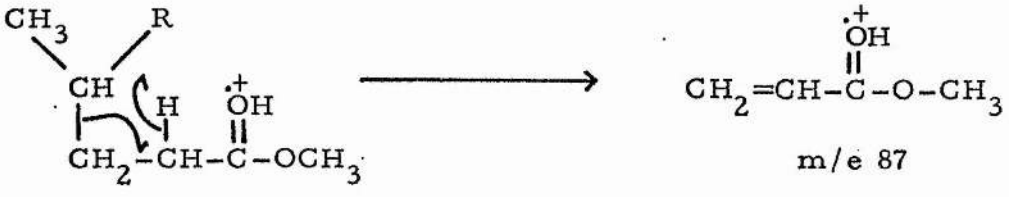
The methyl group at C-10 is amply demonstrated by the expected fragments at m/e 199 and 227. Most methyl esters with methyl side chain at C-10 in addition to a peak at m/e 199 exhibit characteristic peaks at m/e 167 (MeOH elimination) followed by loss of water, to give m/e 149.

Fragments at m/e 269 and 297 indicate a methyl side chain at C-14. The mass spectrum of the P_{19} methyl ester is shown on page 145. The very low peak intensities in the high region part of this spectrum have previously been commented on[114].

d) Mass spectrum of the P_{16} methyl ester



The molecular ion (270 (6%)) indicates the methyl ester of a saturated C_{16} acid and this is confirmed by the acylium fragment at m/e239 (M-31), indicative of methyl esters. The base peak at m/e 87 is that expected of an ester with a 4-methyl substituent. Another



fragment characteristic of a 4-methyl group is observed at m/e 213 (M-57) and indicates the loss of a four carbon fragment (C-2 to C-4

and attached methyl group). The fragment ion at m/e 115 is also indicative of a methyl side chain at C-4.

Evidence for the 8-methyl substituent is afforded by the fragment ions at m/e 157 and 185. The ion at m/e 185 undergoes a series of fragmentations to produce ions at m/e 153 (loss of CH_3OH) and m/e 135 (further loss of water).

Fragment ions at m/e 227 and 255 are evidence for a 12-methyl substituent. The intensities of those two peaks are low and equal as observed by Gason and Graham[114]. The ion at m/e 255 loses methanol to produce ion at m/e 223, which sequentially loses water to give ion at m/e 205. The mass spectrum is shown on page 145.

It is concluded therefore that this ester is methyl 4, 8, 12-trimethyltridecanoate.

e) Structural identification by glc

Glc analysis on polar and non-polar phases was the second technique used in the structural determination of the P acids. The ECL of branched-chain acids have been reported by Jamieson[115]. Fractions in which are concentrated the phytol-based acids contain three major components (A, C, D) and one minor component (B) with the ECL indicated below, on ApL and SP2340 columns.

	A	B	C	D
ApL (200°)	14.35	15.30	16.45	17.55
SP2340 (185°)	14.10	14.75	15.60	16.90

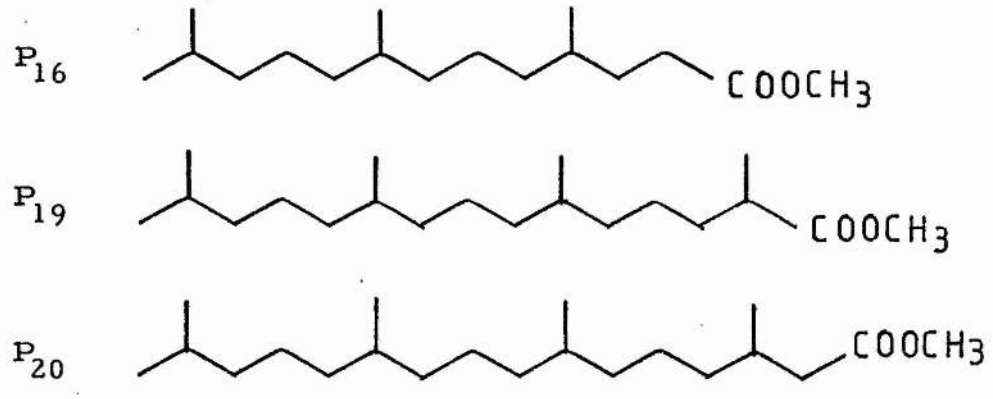
The ApL values agree well with those reported by Douglas et al[116] for P_{16} (methyl 4, 8, 12-trimethyltridecanoate), P_{17} (methyl 5, 9, 13-trimethyltetradecanoate), P_{19} (methyl 2, 6, 10, 14-tetramethylpentadecanoate), and P_{20} (methyl 3, 7, 11, 15-tetramethylhexadecanoate).

The ECL are of interest in that each ester has a value much lower than that of the straight-chain alkanoate of equivalent molecular weight. This results from the fact that the FCL (fractional chain length) of branched methyl groups, though it varies somewhat with the position of branched methyl groups in a carbon chain, is unexpectedly low. For example, the four branched methyl groups in P_{19} and P_{20} add only 1.41 and 1.50 units respectively to the chain length on ApL. On the SP2340 column these figures are even lower at 0.60 and 0.90 respectively.

On polar stationary phases ECL values are affected by operating temperature and polarity of the stationary phases[100, 114] while on non-polar phases ECL values of multi-branched acids may be altered by the concentration of the components on the column.

f) PMR spectra of P_{16}, P_{19} and P_{20} methyl esters

The isoprenoid esters (P_{16}, P_{19} and P_{20}) were also characterised using proton magnetic resonance spectroscopy. Samples were analysed in a Bruker 360 MHz instrument, and the results are summarised.



(1) All three spectra show a 3-proton singlet at 3.66 δ ($COOCH_3$, the carbomethoxy group).

(2) Methyl signals: these are always attached to a CH group and appear as doublets.

P_{16}: They are partially superimposed and confined to the 0.83-0.87 δ region

P_{19}: One doublet is shifted to 1.14 δ, the rest are in the 0.82-0.87 δ region where they appear as three doublets one of which is of double intensity. The 1.14 δ doublet may result from the 2-methyl group.

P_{20}: One doublet is shifted to 0.94 δ (the 3-methyl group). The rest give rise to two doublets each of double intensity in the 0.83-0.87 δ region.

These results are consistent with a methyl group on C-2 in P_{19}, on C-3 in P_{20}, and in neither of these positions in P_{16} (attachment to C-4 is not proved).

(3) Hydrogen atom(s) attached to C-2 [normally 2.2 δ]. In P_{19}, this is a clear sextent (1H) at 2.44 δ. In P_{16} and P_{20} this appears as a more complex signal at $\sim$2.3 δ.

(4) Hydrogen atom(s) attached to C-3 (normally $\sim$ 1.6 δ] and the remaining CH_2 and CH groups.

All three spectra contain an unexplained (large) singlet at 1.53 δ (P_{16}), 1.53 δ (P_{19}) and 1.54 δ (P_{20}). The possibility of these signals originating from an impurity cannot be ruled out. All the spectra contain complex signals between 1.0 and 1.7 δ which must be due to residual CH_2 and CH groups but these

cannot be assigned.

2.2.4 Summary

The assignment of structure of the isoprenoids (P_{16}, P_{19} and P_{20} esters) was based on the following experimental evidence:

(1) The method of isolation of these P esters (urea fractionation) concentrated branched-chain and cyclic esters only. The latter were successfully and completely freed from the former leaving pure isoprenoid ester (> 99%).

(2) The glc behaviours of these esters (P esters) on polar and non-polar columns are consistent with published data. P_{16}, P_{17}, P_{19} and P_{20} esters were tentatively identified. Their ECL before and after hydrogenation remained unchanged showing that these esters are saturated compounds.

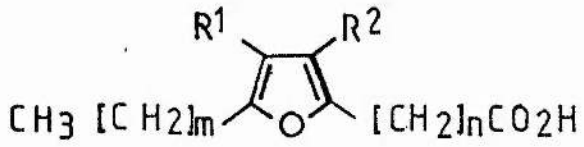
(3) The mass spectra of P_{16}, P_{19} and P_{20} showed their respective molecular weights. The methyl side chains at various positions in the chain were established in each case by their fragmentation patterns.

(4) The proton magnetic resonance spectra of the P_{16}, P_{19} and P_{20} esters finally showed them to be isoprenoid.

2.3 FURANOID ACIDS

2.3.1 Introduction

The widespread occurrence of furanoid acids in fish lipids has been reported and unequivocally confirmed. Morris et al[26] first reported the existence of a fatty acid with a furan ring. They isolated and identified 9,12-epoxyoctadeca-9,11-dienoic acid (FE), from the seed oil of Exocarpus cupressiformis. Glass et al later isolated[22] and identified[23] a group of eight related furanoid acids from Northern Pike (Esox lucius) liver and testes lipids. Structures of some of the naturally occurring furanoid acids are summarised below.



	FE	F1	F2	F3	F4	F5	F6	F7	F8	F9
R^1	H	Me	H	Me	Me	H	Me	Me	H	Me
R^2	H	Me	Me	Me	Me	Me	Me	Me	Me	Me
m	5	2	4	4	2	4	4	2	4	4
n	7	8	8	8	10	10	10	12	12	12

The furan ring is the characteristic feature of the furanoid acids with the double bonds of the heteroaromatic ring distinct from those of olefinic bonds. There are at present two series of this acid obtained from fish with m=2 or 4, and chain-lengths ranging from C_{16} to C_{22}. The furan ring in the FE acid has no methyl substituents, while F acids from fish oils have one or two methyl substituents on

the ring. A recently discovered furanoid acid[27] from rubber latex (Hevea brasiliensis) was identified as 10,13-epoxy-11-methyloctadeca-10,12-dienoic acid (ie. identical with F2).

Since the discovery of furanoid acids in fish oils by Glass et al[23] interest in these novel acids has been shown by a number of workers. Gunstone and Wijesundera[24], having surveyed the existence and distribution of furanoid acids in several species of fish, reported that furanoid acids in the liver of these fish existed at a level of 1 to 4% with the F6 (C_{22}) acid usually dominant. They observed a marked increase in the proportion of furan acids in starved cod liver lipids. In a quantitative analysis of furanoid acids in crude and refined cod liver oil, Scrimgeour[25] found that these acids were present at a level of 1% or less, a figure similar to that reported by Gunstone et al[24].

Isolation and identification of furanoid acids from fish oils and other sources, have been carried out using different approaches. Glass et al[23] separated the lipids into classes by tlc (silica gel H) and carried out alkaline-catalysed esterification of each class. Acid-catalysed hydrolysis is undesirable because furans may be unstable under acid conditions. After hydrogenation of the more conventional unsaturated acid Glass et al[23] used argentation chromatography to separate and enrich the furans of the Northern Pike (Esox lucius). These represented 25% of the total liver lipids of which 90% of the acids were bound in cholesteryl esters. At these high levels of occurrence, isolation and identification of these acids by the methods outlined above do not present much difficulty.

Scrimgeour[25], in a quantitative analysis of furanoid acids in cod liver oil, used silver ion tlc and urea crystallisation with added squalene as internal standard. In another study of long-chain furanoid acids, Wijesundera[138] used urea crystallisation followed by preparative silver ion tlc of the mother liquor. He isolated and identified F1 to F7, observed the presence of F8 and F9, and suspected the presence of small quantities of unsaturated furanoid esters, which had been noted earlier by Glass et al[22]. In a recent paper by Glass et al[28] the furanoid acids, F5 and F6 were synthesised from 3,4-bis(acetoxymethyl)furan and pentanoic acid anhydride.

In the present study, a detailed examination of the furanoid acids of cod liver oil was undertaken.

2.3.2 Isolation

Isolation of furanoid esters was carried out using urea fractionation, silver ion column chromatography and silver ion tlc. Methyl esters were obtained by base-catalysed transesterification of the cod liver oil. A scheme for the separation and isolation of the furanoid esters is similar to that in Scheme II, section 2.2

The methyl esters were crystallised with urea and methanol using 2 g of urea per g of ester. The esters remaining in the mother liquor were submitted to a second treatment with urea (5 g per g of ester). The second liquor was eluted from a column of silica containing silver nitrate, with a range of solvents of increasing polarity. The eluted fractions, containing the branched-chain and furanoid esters, made up about 15% of the

second mother liquor. Polyene esters were not removed from the column. Based on the results of glc analysis and quantity of esters recovered, the eluted material was combined into four fractions (A'-D'). The A' fraction was applied to a silica column and eluted with successive solvents of increasing polarity (PE1, PE2, PE5 followed by PE10, PE20, PE50 and diethyl ether). The progress of elution was monitored by tlc. The bulk of the methyl esters was concentrated in fractions 5 and 6 and later combined (Table 5). This combined fraction contains mainly phytol-based esters (70%) and furanoid esters (22%).

Preparative argentation tlc was employed as the next purification step to separate branched-chain esters from traces of monoenes and polyenes, and to separate the P esters from the cyclic furanoid esters. Samples of about 50 mg were applied to each plate (0.5 mm thickness) which was developed with PE7.5 . An authentic sample of phytanate ester helped to locate the position of the P esters. Chromatographic analysis (glc) showed the presence of saturated esters and hydrocarbons in band 1, isoprenoid esters (99%) in band 2, furanoid esters in band 3, and monoene esters in band 4. The final band still at the point of application was probably polyene esters and was discarded.

On silver ion tlc plates, developed with PE5, PE10, or benzene/hexane (1:1), furanoid esters seem relatively unretarded. They migrate just ahead of the monoenes and somewhat behind the saturated straight-chain and the isoprenoid esters. The higher homologues of the furanoid esters move further than the lower homologues. For example, F1 (C_{18}) and F2 (C_{19}) were found to

occupy the same band as the monoenes while F5 (C_{21}) and F6 (C_{22}) moved closer to the isoprenoid esters. The glc analysis of the furan band is shown in Table 5. F1 and F3, present in smaller amounts than the rest of the furans (F2-F6), can now be recognised. The total amount of the furanoid esters rose from 22% to about 62%.

Preparative glc was finally used to isolate individual furanoid esters prior to structural determination by mass spectrometry. Preparative glc was carried out as described in the general experimental section except that the oven temperature was varied between 170-190°C. The temperature of the outlet tube was set at about 200° as higher temperatures resulted in deterioration and loss of furanoid esters. This latter factor was mainly responsible for the failure to achieve near 100% purity of the separated components. However over 85% purity was obtained in each of the isolated components.

Some minor components which could not be isolated in pure form were identified tentatively on the assumption that they belong to a homologous series. Glass et al[23] originally identified only one furanoid acid unambiguously (F6) and assumed that the remainder had a homologous relationship to this acid. Some additional structures are confirmed in this study.

Table 5. Component acids (%, wt) of the furan band (SP2340, 185°)

Assignment	
C11 br	0.3
Isoprenoid acids	
P_{16}	7.9
P_{20}	5.1
Other branched-chain esters	
C17 br chain	3.9
C18 br/18:1	12.9
C19 br chain	1.9
C20 br chain	6.4
Furanoid esters	
F1	2.8
F2	16.8
F3	2.8
F4	7.5
F5	9.7
F6	22.2

2.3.3 Structural identification

a) Identification by glc behaviour

Tentative identification of the furanoid esters was achieved by glc on polar (SP2340 at 186°) and non-polar (ApL at 200°) columns. Before chromatographic analysis, the furan band was purified by further preparative argentation tlc to concentrate the furanoid esters still more. The furans F8 and F9 which had remained insignificant then appeared on the glc trace. On the SP2340

column the F1-F9 furanoid ester components (ECL 20.5-25.8) were well separated. On the same column under identical conditions the C_{16}-C_{20} monoene standards gave ECL, of 16.6, 18.4, 20.4 and 22.3 The F1 ester coincided with 20:1, while F4 coincided with 22:1. A series of esters of yet unidentified structures of ECL 16.7, 17.7, 18.8, 19.8 and 20.8, accompanied the furan band and complete separation of these esters from the furanoid esters were not obtained by silver ion chromatography.

It is important that the monoenes be eliminated before accurate ECL of the furans are determined. As already mentioned, complete elimination of these monoenes, which constitute the major class of the cod liver oil was achieved using urea fractionation and argentation chromatography. The ECL (SP2340) reported in this study were in good agreement with those published in the literature on other polar columns[23-25, 117-119] and the observed values were reasonably consistent over the period of study of several months.

The furanoid band was also analysed on ApL column (3.5%). The percentage composition of the various furanoid esters was fairly similar to that obtained with SP2340 column. Assignment of ECL to the various furanoid esters was difficult for the following reasons. A non-polar column such as the ApL separates substances mainly according to chain length. But the furanoid esters may not necessarily be separated according to chain length due to effects of the substituents. There were no ECL of these esters on non-polar columns in the literature for comparisons to be made. Assignment of ECL on ApL column was therefore based on percentage composition of the components on SP2340 and ApL columns. The changes in ECL between various furanoid esters on both ApL and SP2340 were consistent and in

accord with the structures assigned by others and confirmed in this report by mass spectrometry. The ECL of the furanoid esters on polar and non-polar columns are as detailed in Table 6.

Table 6. ECL of the furanoid esters on SP2340 and ApL columns

10% SP2340 (186°) ECL	3.5% ApL (200°) ECL	Assignment
20.5	17.5	F1
21.3	18.5	F2
22.1	19.2	F3
22.5	19.5	F4
23.3	20.6	F5
24.0	21.0	F6
24.4	21.4	F7
25.2	22.5	F8
25.8	22.8	F9

As expected the difference in ECL between homologues having two additional carbon atoms is close to 2.0 (Table 7). When two esters differ through having one or two methyl groups in the furan ring the additional methyl group has an FCL (fractional chain length) of about 0.8 on SP2340 and 0.4-0.7 on ApL. As previously noted with the phytol-based esters, branched methyl groups have an FCL below 1.0 . It is however noteworthy that a synthetic C_{18} 10,13-furan prepared in this laboratory by Gunstone et al[118] had similar FCL to its mono-methyl analogue F2 (C_{19}) and these two furans could not be separated on polar columns.

The figures in Table 7 show the differing glc behaviour of isomeric furanoid esters. A shift of two carbon atoms from the m=2 to m=4 series of compounds produces a difference of about 0.4 in the ECL. The observation in this study is in line with the broader study of isomeric furanoid esters synthesised by Lie Ken Jie et al[120,121].

It follows that the glc behaviour of the furanoid esters depends on the total chain-length, the number of branched methyl groups, and the position of the heterocyclic ring within the chain.

From the glc behaviour of the isolated furanoid esters on both polar and non-polar columns, and the ECL of these esters, it is likely they are furanoid esters. These ECL are consistent with and similar to published results. The identification of these esters is however tentative and mass spectrometry of these esters is required to confirm their identity.

b) Mass spectrometry

The furanoid esters lend themselves to examination by mass spectrometry since they give a simple fragmentation pattern. In addition to the molecular ion, they produce three significant ions resulting from cleavage a and/or b.

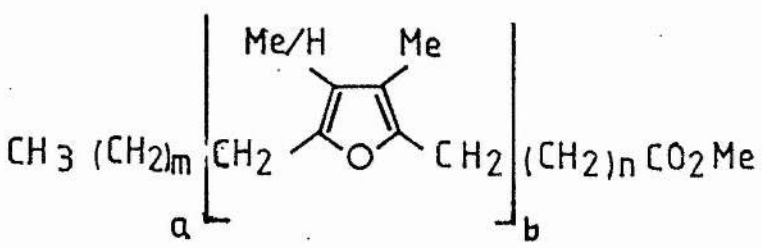


Table 7. ECL increments of furanoid esters on SP2340 and ApL columns

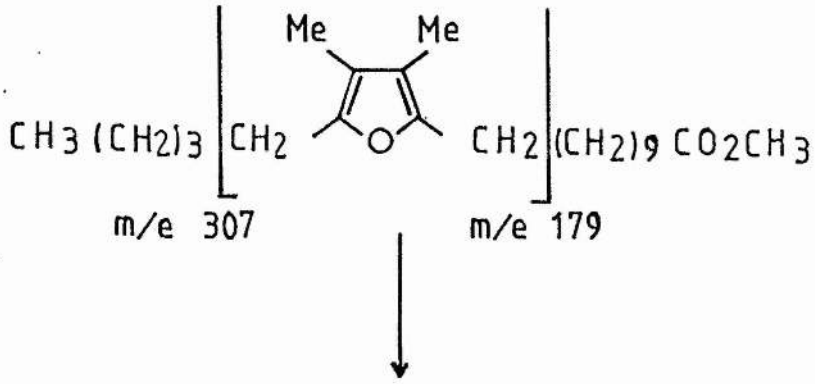
	ECL	
(1) Homologues	SP2340	ApL
F1 - F4	+ 2.0	+ 2.0
F4 - F7	+ 1.9	+ 1.9
F2 - F5	+ 2.0	+ 2.1
F5 - F8	+ 1.9	+ 1.9
F3 - F6	+ 1.9	+ 1.8
F6 - F9	+ 1.8	+ 1.8
(2) Additional branched methyl		
F2 - F3	+ 0.8	+ 0.7
F5 - F6	+ 0.7	+ 0.5
F8 - F9	+ 0.8	+ 0.4
(3) Isomers		
F3 - F4	+ 0.4	+ 0.3
F6 - F7	+ 0.4	+ 0.4

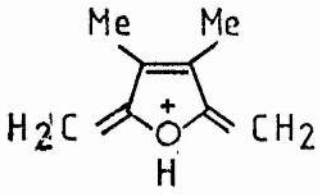
These fragmentation patterns indicate the structure of these esters very clearly, except that the position of a single methyl group on the furan ring is not fixed. The conclusions in this respect are therefore based on those of Glass et al[23] who obtained other evidence on this point.

(i) F6 ester

The mass spectrum of this ester (page 151) showed a large molecular ion of m/e 364 (64%), a base peak at m/e 179 and significant peaks at m/e 365 (18%, M+1), 307 (57%, a), and 123

(21%, furan fragment). These fragments show that F6 has the structure indicated below. The ion of m/e 123 results from two fragmentations and migration of hydrogen to form the furan fragment indicated below.

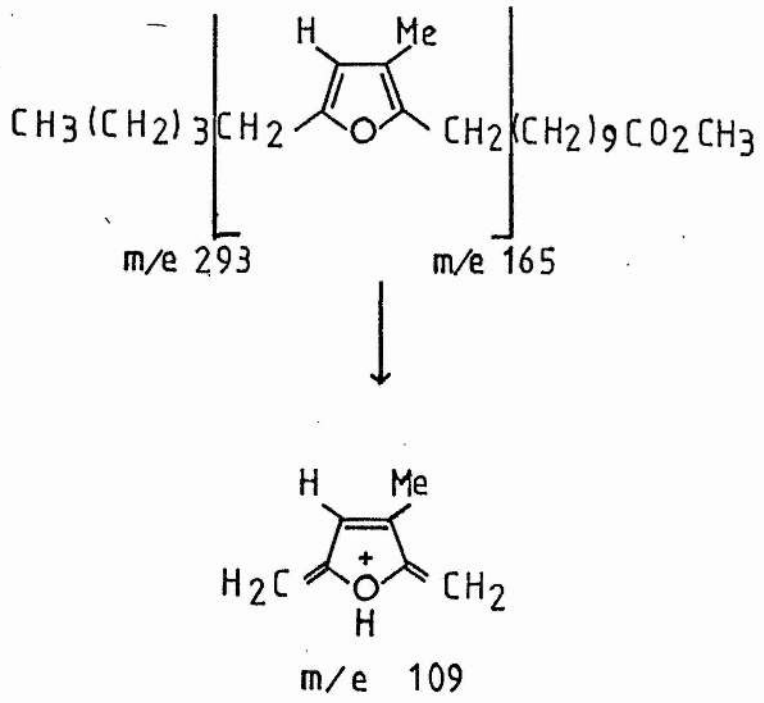




m/e 123

(ii) F5 ester

The mass spectrum of this ester (page 151) contained a molecular ion of m/e 350 (53%), a base peak at 165, and characteristic peaks at m/e 293 (17%, a) and 109 (19%). The fragmentation of F5 ester is shown overleaf.



(iii) F3 and F4 esters

The preparative glc column employed (ApL) separated the F esters according to chain length, and both F3 and F4 were collected as a single component. The separation between these two did not improve by lowering column temperature and/or flow rate of the carrier gas.

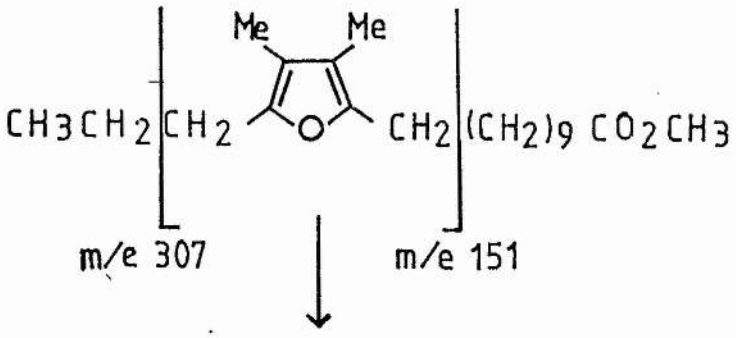
The two esters were later resolved by a GC-MS technique.

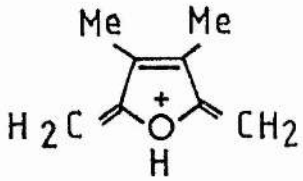
1. F4 ester

The mass spectrum of this ester (page 150) displayed a molecular ion of m/e 336 (47%) and an M+1 ion at m/e 337 (10%). The base peak occurred at m/e 151 and characteristic ions at m/e 307 (42%) and 123 (18%). The fragmentation pattern is shown on page 62.

2. F3 ester

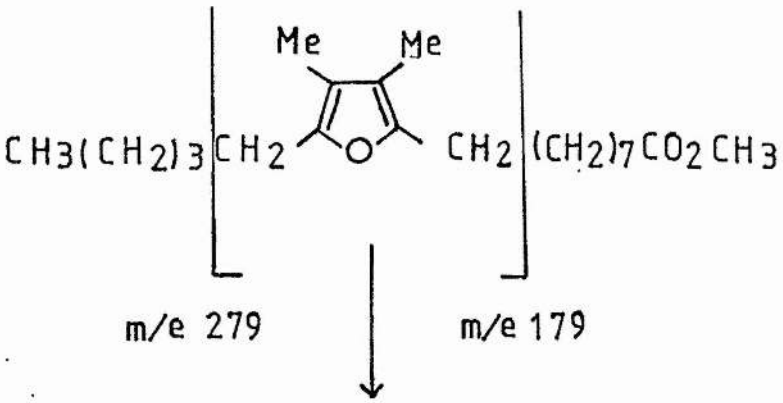
The mass spectrum of this ester (page 150) showed a large molecular ion at m/e 336 (47%), a base peak at m/e 179 and

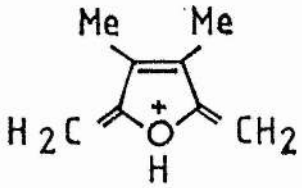




m/e 123

characteristic ions at m/e 279 (41%) and 123 (19%). The fragmentation pattern is shown below.

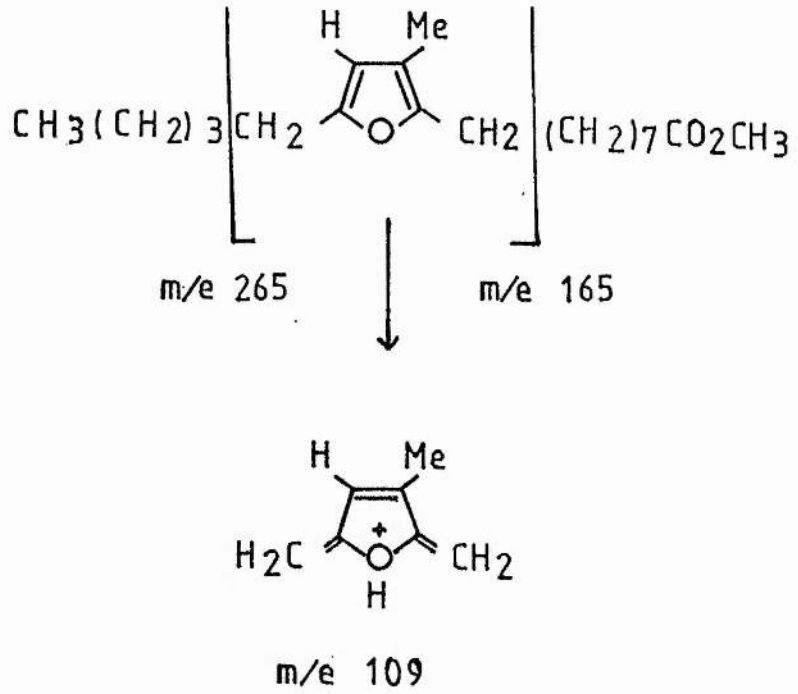




m/e 123

(iv) F2 ester

The mass spectrum of the F2 ester (page 148) showed a large molecular ion of m/e 322(57%), a base peak at m/e 165(b) and peaks at M+1 (12%), m/e 265(22%,a) and m/e 109(22%, a-alkyl ester). These fragments show that F2 has the structure indicated below.



The mass spectra of the F esters (F2-F6), so far discussed, are similar to those reported elsewhere[23]. The characteristic ions in the mass spectra of the furanoid esters (F2-F6) are summarised in table 9.

Esters F1, F7, F8 and F9 present at low levels were not individually isolated. Therefore only tentative identification by glc on polar and non-polar columns is possible.

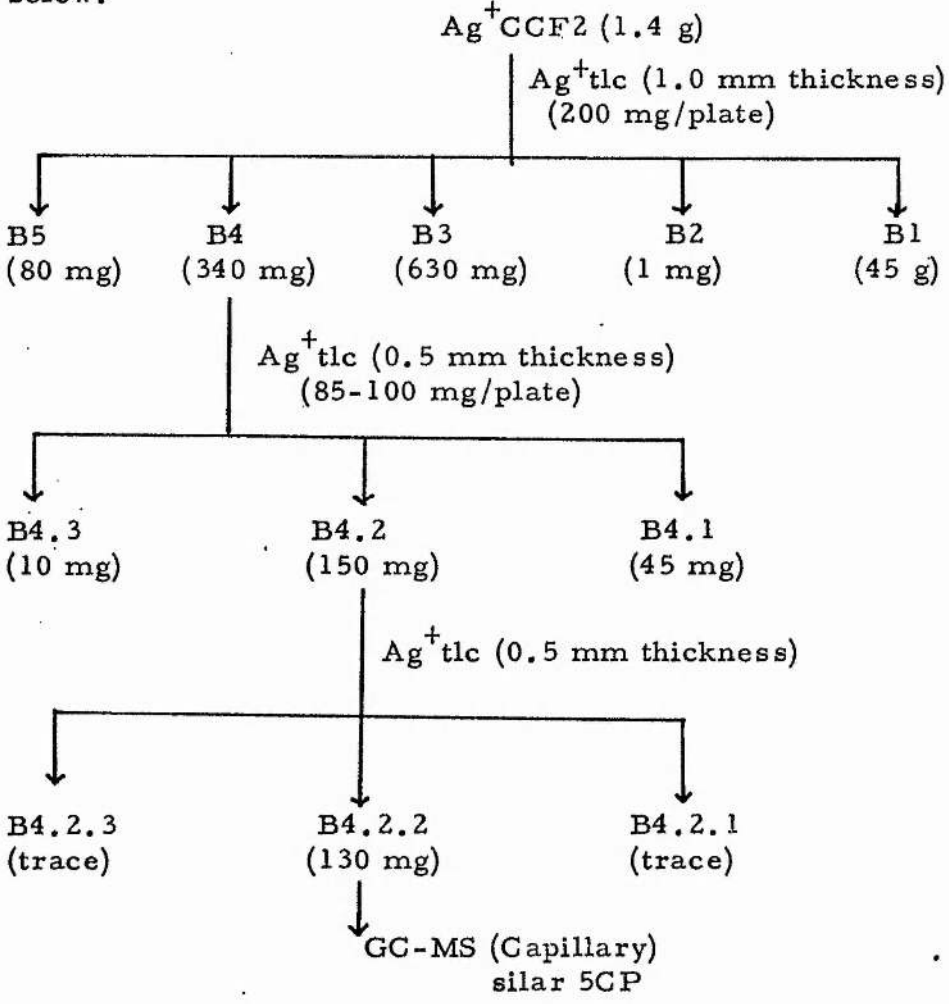
2.3.4 GC-MS studies of a furan band

At a later date it became possible to make a GC-MS study of the furan esters using a 25 m Silar 5CP capillary column linked to the mass spectrometer. Spectra were recorded at 70 ev on

native esters and on their perhydro derivatives. Adoption of GC-MS studies of the isolated furan band was necessary for the following reasons. The earlier method using column glc (ApL) after purification by preparative glc failed to isolate some of the individual furanoid esters present at low concentration. More important, certain esters of unfamiliar ECL could not be identified by their ECL alone. The GC-MS (capillary) method therefore seemed the obvious technique to use to isolate and identify the various components.

a) Isolation

Isolation of part of the furan band was carried out as shown below.



Fraction Ag^{+}CCF2, as outlined in section 2.4, Scheme IV, obtained by a combination of urea fractionation, silver ion chromatography (column) and column chromatography (silica), was further separated on silver ion tlc plates developed in PE7.5 . Chromatographic analysis (glc) showed the presence of hydrocarbons in band 1, saturated esters in band 2, isoprenoid esters in band 3, a mixture of unfamiliar esters and some furanoid esters in band 4, while band 5 contained mainly furanoid and some monoene esters.

Band 4 was subjected to further preparative silver ion tlc to give first band 4.2 and then band 4.2.2 . This was finally subjected to a GC-MS study (Table 9). The glc compositions of bands 4.2 and 4.2.2 are summarised in Table 8.

An aliquot of band B4.2.2 was hydrogenated employing 10% palladium on charcoal and the perhydro product was analysed on SP2340 (Table 7).

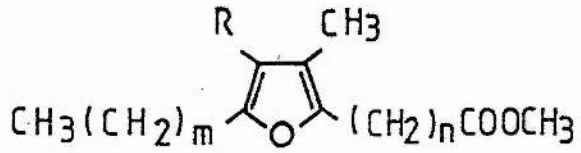
b) Characterisation of furanoid esters

GC-MS study of the B4.2.2 ester band showed this to consist of saturated branched-chain esters (see section 2.2), unsaturated branched-chain esters (see section 2.4) and a series of furanoid esters. These are considered to have the structures listed in Table 9.on the basis of the molecular and fragment ions. Two unidentified furanoid esters (mass spectra, pages 148 and 149) may be olefinic compounds corresponding to those already suspected to occur in fish oils (Gunstone et al[24], Glass et al[23]).

Table 8. Component acids (% wt) of bands B4.2, B4.2.2 and perhydro derivative of B4.2.2 (SP2340, 185°)

Assignment	B4.2	B4.2.2	B4.2.2 (perhydro derivative)
12:0	-	trace	trace
P_{16}	2.7	1.9	4.3
P_{17}	-	0.5	-
C15 br	0.4	0.8	-
P_{19}	0.7	-	0.2
16:0 br	-	1.0	28.2
C16 br	0.9	1.6	-
16:1/C16 unsat. br	1.5	3.6	-
P_{20}/17:0 br	4.7	-	-
C17 br	0.9	0.4	-
C18 br	-	-	2.9
18:2 br	23.1	22.2	-
C19 br	-	-	0.8
19:2 br	3.0	1.4	-
20:0 br	1.5	0.5	3.5
20:2 br	11.7	4.5	-
C20 unsat. br	-	-	7.3
F1	-	-	1.9
F2	23.3	21.6	22.7
F3	1.6	2.7	6.0
F4	12.4	13.4	15.7
F5	6.9	7.0	0.6
F6	4.7	15.1	5.8
F7 ?	-	1.1	-
F8 ?	-	0.7	-
F9 ?	trace	trace	-

Table 9. Structural characteristics of furanoid esters isolated from band 4.2.2, showing molecular ions and characteristic fragments

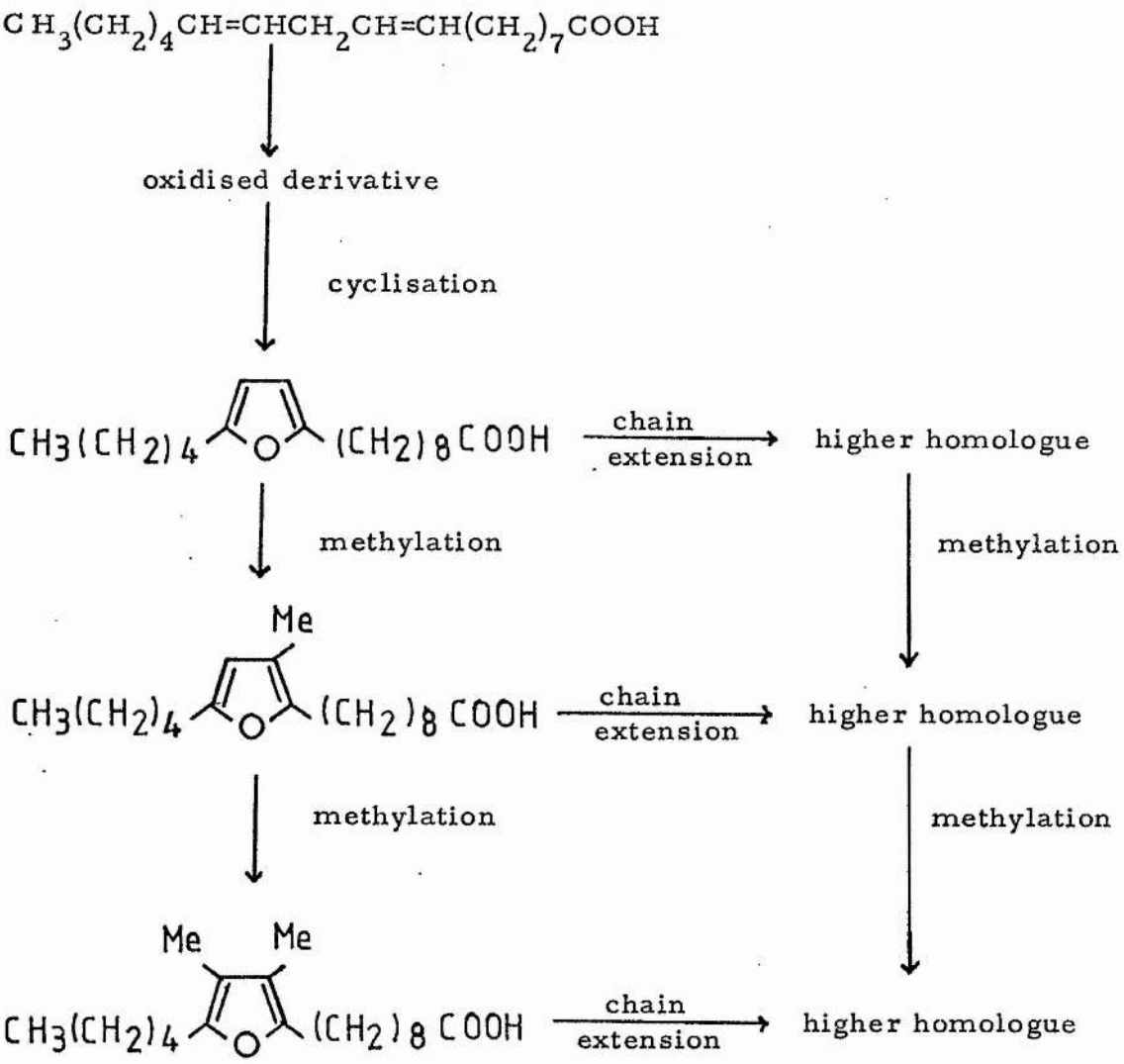


Total no. of carbon atoms in the acid	m	n	R	Assignment	M^+	a	b	Furanoid fragment	Mass spectra (page no.)
13	3	1	H	*	238(44%)	181(40%)	165(100%)	109 (8%)	161
17	4	6	H	*	294(25%)	237 (8%)	165(100%)	109(35%)	146(a)
17	2	8	H	*	294(50%)	265(16%)	137(100%)	109(17%)	146(b)
18	4	6	Me	*	308(32%)	251(52%)	179(100%)	123(23%)	147
18	2	8	Me	F1	308(38%)	279(43%)	151(100%)	123(12%)	147
19	4	8	H	F2	322(57%)	265(22%)	165(100%)	109(27%)	148
19	2	10	H	*	322(24%)	293 (8%)	137(100%)	109(11%)	149
20	4	8	Me	F3	336(47%)	279(41%)	179(100%)	123(19%)	150
20	2	10	Me	F4	336(47%)	307(42%)	151(100%)	123(18%)	150
21	4	10	H	F5	350(53%)	293(17%)	165(100%)	109(19%)	151
22	4	10	Me	F6	364(64%)	307(57%)	179(100%)	123(21%)	151

Note: The furanoid esters marked with asterisks are reported for the first time.

2.3.5 Biosynthetic speculation

There is no convincing experimental evidence concerning the biosynthesis of furanoid acids now discovered in the plant and animal kingdom though there has been some speculations[122-130] which include the dehydration of endoperoxides[131] and photo-oxidation of linoleate[132]. Relating the position of the furan ring to the end methyl group then the furanoid acids belong to the n-4, n-6 and n-7 series and it is possible that these are derived from the Δ9, 12 dienes of C_{16} and C_{18} acids as in the following scheme:



2.4 OTHER BRANCHED-CHAIN ACIDS

2.4.1 Introduction

The discovery and characterisation of an unsaturated branched-chain fatty acid in fish lipids was first reported by Sano[133] who isolated 7-methylhexadec-6-enoic acid from whale oil. Ackman et al[134] found the same acid to be a minor component in the lipids of marine animals and also identified 7-methylhexadec-7-enoic and 5-methyltetradec-4-enoic acids in the sperm whale[135]. Recently Pearce and Stillway[136] isolated and identified 7-methylhexadec-7-enoic acid from the spadefish liver oil.

The occurrence of these unusual fatty acids is not confined to aquatic sources. Gerson et al[137] recognised 11-methyloctadec-11-enoic among other branched-chain acids from the lipid of Rhizobium cultured under controlled conditions.

The origin of these unusual acids in aquatic animals is not clear. Ackman et al, however, have suggested that the occurrence of 7-methylhexadec-7-enoic acid from the Sunfish Mola mola[134] (Linnaeus), marine turtles[138] and jelly fish[139] may be dietary; while Karlsson et al[140] have suggested that some unusual fatty acids from marine origin may arise from an unusual diene sphingosine derivative by metabolic conversion. They identified this base as D-erythro-1,3-dihydroxy-2-amino-9-methyl-trans-4,trans-8-octadecadiene.

These unsaturated branched-chain fatty acids are isolated by techniques similar to those used for the isolation of other branched-chain acids. Sano[133] used urea fractionation, fractional distillation, a second urea fractionation and column chromatography on $AgNO_3$-Al_2O_3. Ackman[134] and Pascal and Ackman[135] adopted urea

fractionation, silver ion tlc of the mother liquor and preparative glc.

The presence of these unusual acids in many aquatic sources prompted the search for similar acids in cod liver oil.

2.4.2 Isolation of a concentrate of branched-chain acids for GC-MS examination

(a) Isolation of concentrate

In this project isolation of unsaturated branched-chain esters was carried out employing urea fractionation, silver ion column chromatography and argentation tlc. Details of the isolation procedure are outlined in section 2.2 page 39, scheme III. The mother liquor ($AgCCF_2$) was submitted to a series of silver ion tlc separations to obtain a band F4.2.2 as shown in section 2.3.4 (a), page 64.

Band F4.2.2 contains furanoid esters and some unsaturated branched-chain esters (see Table 8, section 2.3). Notable amongst these is a series of esters of unfamiliar ECL values which differ by one ECL unit. On a polar column (SP2340) esters of ECL 18.8, 19.8 and 20.8, predominated over other branched-chain esters. Attempts to free this band of the furanoid esters were unsuccessful.

(b) GC-MS of hydrogenated concentrate (band F4.2.2)

A GC-MS study of the concentrate (band F4.2.2) after hydrogenation was carried out using a 25 m Silar 5CP capillary column linked to the mass spectrometer. The study provided spectra of 16 components. The first seven were found to be mono or dimethylalkanoates and the remainder proved to be hydrogenated furan esters. Of the seven spectra relating to the open-chain

compounds, the largest peaks corresponded to methyl esters of 7-methylhexadecanoic and 7,9-dimethylhexadecanoic acids. These were preceded by methyl 4,8,12-trimethyltridecanoate (see section 2.2, page 45), 5-methyltetradecanoate, 5,7-dimethyltridecanoate and 5,7-dimethyltetradecanoate, and followed by methyl 9-methyloctadecanoate, 7,9-dimethyloctadecanoate and 9,11-dimethyloctadecanoate.

Mass spectrum 26 (page 155) shows a major molecular ion at m/e 256 and smaller signal at m/e 270. The spectrum relates mainly to methyl 5-methyltetradecanoate along with 5,7-dimethyltridecanoate. The structure of the major component is based on the molecular ion (m/e 256), the base peak at m/e 74 and significant ions of m/e 241 (M-15), 227 (M-29), 213 (M-43, 206 (M-50) and 180 (M-76). Fragment ions of m/e 101 and 129 (which losses 32 atomic mass units to give an ion at m/e 97) indicate the presence of a methyl side chain at C-5.

Mass spectrum 27 (page 156) shows a major molecular ion at m/e 270 and a minor signal at m/e 256. The spectrum relates mainly to methyl 5,7-dimethyltetradecanoate. The structure is based on the molecular ion (270), the base peak at m/e 74 and significant ions at m/e 255 (M-15), 227 (M-43), 220 (M-50) and 194 (M-76). Fragment ions at m/e 101, 129, 143 and 171 each of which loses 32 atomic mass units to give ions at m/e 69, 97, 111 and 139 respectively, indicate the presence of methyl side chains at C-5 and C-7. This is confirmed by the ketene ions at m/e 121 and 139.

Mass spectrum 28 (page 157) shows molecular ions at m/e 284 (major) and m/e 298 (minor). This fraction is mainly 7-methylhexadecanoate based on the molecular ion (m/e 284), base peak (m/e 74), the second largest peak (m/e 87) and fragment ions of m/e 129 and 157 each of which lose 32 atomic mass units to give ions of m/e 97 and 125 respectively.

Mass spectrum 29 (page 157) displayed a molecular ion at m/e 298. The fragment ions at m/e 129 and 157 each of which loses 32 atomic mass units to give ions at m/e 97 and 125 respectively indicate a methyl side chain at C-7. Other fragment ions at m/e 171 and 199 which in turn lose 32 atomic mass units to give ions at m/e 139 and 167 respectively, indicate a C-9 methyl side chain. The spectrum is that expected of a methyl 7, 9-dimethylhexadecanoate.

Mass spectrum 30 (page 158) shows molecular ions of m/e 312 and 326 with the latter being the major component. The minor component produces fragment ions which show it to be 9-methyloctadecanoate. The 9-methyl substituent is indicated by fragment ions at m/e 157, 158 and 159 and at m/e 185, 153 (185-32) and 135 (153-18). The major component (M, 326) with methyl side chains at C-7 and C-9 is confirmed by characteristic ketene ions at m/e 135 and 153 and is therefore a methyl 7, 9-dimethyloctadecanoate.

Mass spectrum 31 (page 159) shows a molecular ion of m/e 326 and relates to methyl 9, 11-dimethyloctadecanoate. The base peak occurred at m/e 74 and significant ions were observed at m/e 87, 101, 115, 129, 130 and 143. Characteristic fragments at m/e 157, 185, 199 and 227 each of which lose 32 atomic mass units to give ions of m/e 125, 153, 177 and 195 respectively, indicate the presence of methyl side chains at C-9 and C-11.

The separation of these methyl-substituted isomers on the Silar 5CP capillary column has not been complete. In most cases the spectrum indicates the presence of two components with molecular weights differing by 14 atomic mass units suggesting that esters with one methyl substituent are eluted coincidently with those with two methyl substituents. However, the detailed glc behaviour of monomethyl- and dimethyl-substituted esters on Silar 5CP column is not known.

A summary of the results of the GC-MS study of the hydrogenated concentrate (band F4.2.2) is shown in Table 10.

c) GC-MS of the concentrate (band F4.2.2) before hydrogenation

The GC-MS study of band F4.2.2 gave the mass spectra 32-46 (pages 160-167). As expected it was not possible to identify the position of the double bond(s) in these esters because the unsaturated centres are labile under electron bombardment[141-143]. It is however possible to determine the degree of unsaturation from the molecular ion and to correlate this with the results obtained on the hydrogenated sample (table 10).

Of these branched-chain esters those based on 7-methylhexadecanoate and 7,9-dimethylhexadecanoate are the major components. It has been possible to obtain a concentrate of the latter and to identify it.

Table 10. GC-MS results of the concentrate (band F4.2.2) before and after hydrogenation

Total no. of carbon atoms in the acid	Hydrogenated sample			Unhydrogenated sample		
	M^+	Assignment	MS page no.	M^+	No. of double bonds	MS page no.
15	256	5-methyltetradecanoate	155	254	1	161
15	256	5,7-dimethyltridecanoate	155	254	1	162
16	270	5,7-dimethyltetradecanoate	156	268	1	163
				266	2	163
17	284	7-methylhexadecanoate	157	282	1	164
				280	2	166
18	298	7,9-dimethylhexadecanoate	157	294	2	165-167
19	312	9-methyloctadecanoate	158			
20	326	7,9-dimethyloctadecanoate	158			
	326	9,11-dimethyloctadecanoate	159			

NB. Mass spectra 32 and 33 (page 160, unhydrogenated sample) may be 11:0 and 14:1 respectively

2.4.3 Isolation and characterisation of U1

a) Isolation of a concentrate of U1

The concentration of U1 (5.6%) present in fraction AgCCF2 was increased by the isolation procedure outlined in Scheme IV. The esters present in AgCCF2 (3.5 g, section 2.2, Scheme III) were eluted from a 30% silver ion column (52 x 9.5 cm) with PE5 (100 ml, 1 mg), a 1:1 mixture of benzene-hexane (100 ml, 2.48 g) and ether (100 ml, 200 mg) (see Table 11).

The F2 methyl ester (U1, 6.5%) was then separated on 20% silver ion tlc plates (1.0 mm thickness, 200 mg/plate) by development with PE5. The fastest moving band (B1) contained mainly P esters, B2 contained furanoid esters and P_{16}, while B3 contained U1 (43.3%). This material, 100 mg/plate, was reapplied to a 20% silver ion tlc plate (0.5 mm thickness) and developed in benzene followed by a second development in the same direction with chloroform/methanol (99:1, v/v). Five different bands were obtained. The composition of bands B3.1 and B3.2 are outlined in Table 11. Band B3.3 contained mainly furanoid ester (F6, 85%). The B3.4 band was insignificant and band B3.5 (near to point of application) was discarded.

The B3.1 band was finally subjected to preparative glc on an ApL column (3.5%) as already described to give a sample of U1 (88% pure by glc) which was used for further characterisation.

b) Gas chromatographic behaviour

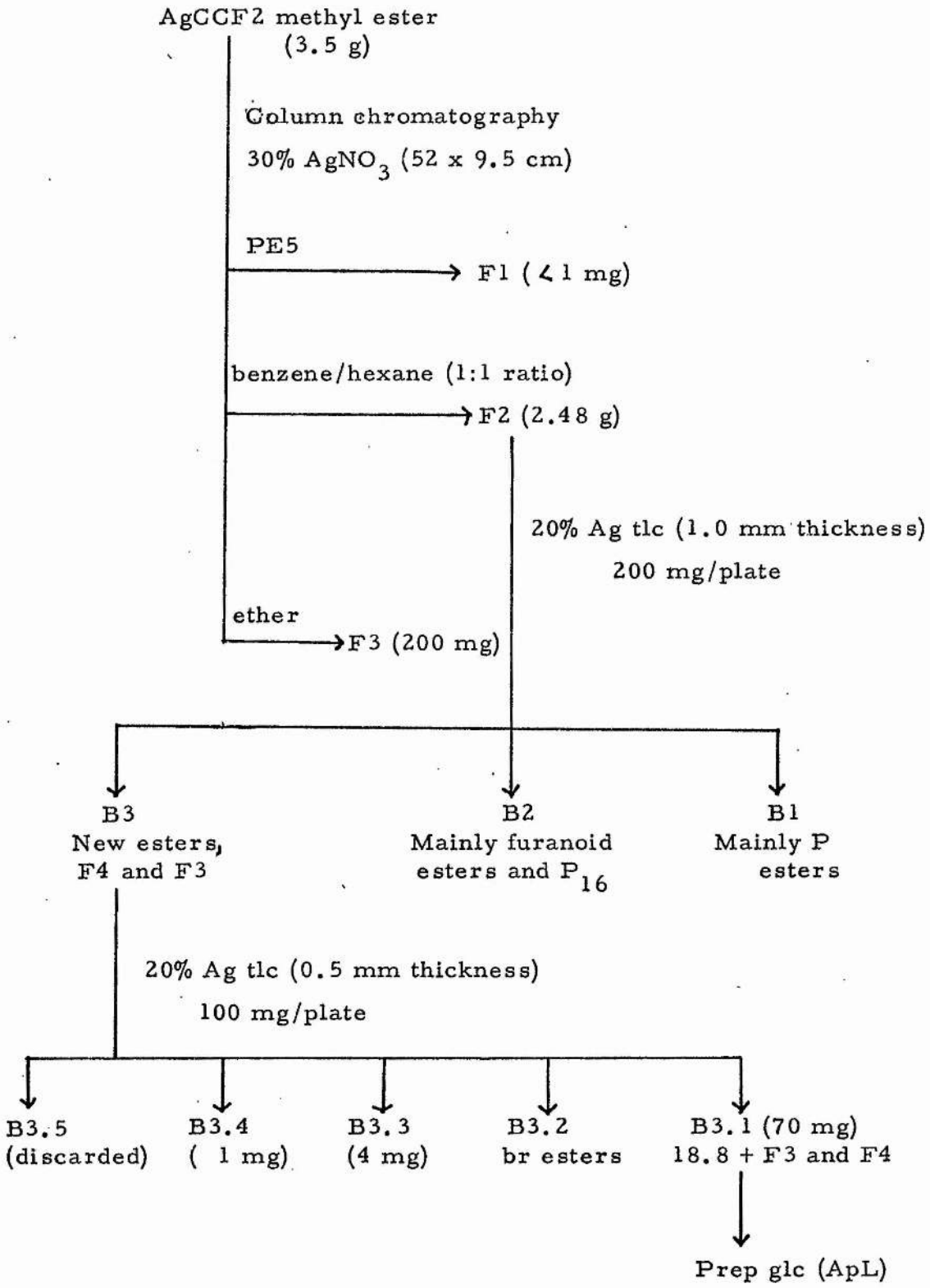
The methyl ester of U1 (88%) gave an ECL of 18.8 (SP2340, 185°). After hydrogenation it showed a double peak with ECL of 16.1 and 16.3. The change in ECL from 18.8 to 16.1/16.3 on hydrogenation is more

than usual for a methylene-interrupted diene system (ca. 1.3-1.5). The separation of the ester into a double peak after hydrogenation may be due to the presence of different diastereoisomers. Further identification of U1 on the basis of the glc behaviour was not possible because these unusual ECL did not fit with Jamieson's[115] retention data.

Table 11. Component acids (%, wt) of fractions F2, F3 and sub-fractions B3.1 and B3.2 (SP2340, 185°).

Assignment	F2	F3	B3.1	B3.2
C_{14} br	12.0	1.8	3.2	1.2
P_{17}	0.8	15.6	-	20.8
C_{15} br	-	-	1.1	-
P_{19}	11.0	-	2.9	23.6
P_{20}	32.0	18.9	7.5	-
C_{17} br	-	9.7	-	11.3
C_{17} br unsaturated	0.6	7.2	1.6	6.0
C_{18} br	0.4	-	-	-
C_{18} br unsaturated	-	18.4	-	24.0
C_{18} br unsaturated (U1)	6.5	-	47.6	-
C_{19} br unsaturated	-	9.6	-	-
C_{18} br unsaturated (diene)	0.8	5.2	4.1	7.5
C_{20} br unsaturated (diene)	1.8	-	7.2	-
Furanoid esters (F1-F6)	(34.1)	13.6	24.8	5.6

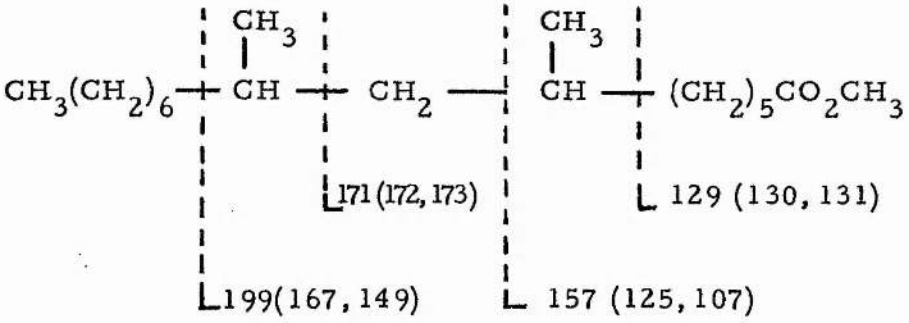
Scheme IV Flow diagram for the isolation of unsaturated branched-chain ester U1



c) Mass spectrum of hydrogenated sample (U1)

Mass spectrometry of the perhydro derivative of U1 was undertaken to determine chain length of the ester and to locate positions of the branched-chains. The general fragmentation behaviour of saturated branched-chain esters has already been discussed in section 2.2, page 34.

The molecular ion of this spectrum (20) (page 152) at m/e 298 indicates a methyl ester of a C_{18} acid, and the base peak at m/e 74 confirms the presence of a methyl ester.



Cleavage of the bond in the fatty acid ester chain on the ester side of a methyl branch gives rise to ions at m/e 129, 130, and 131, and on the other side of the methyl branch to ions at m/e 157, 125 (157-32), and 107 (125-18). These ions confirm the presence of a methyl side chain at C-7.

Fragment ions at m/e 171, 172, 173 and 199, 167 (199-32) and 149 (167-18) indicate the location of a second methyl side-chain at C-9.

The available evidence so far suggests that the ester U1 is an unsaturated derivative (-4H) of 7,9-dimethylhexadecanoic acid.

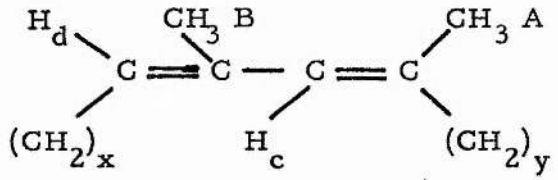
d) Spectroscopic information, uv, ^{1}H nmr

(i) Uv spectroscopic spectrum of U1

The uv spectrum of U1 in cyclohexane solution had a maximum absorption at 230 nm. This wavelength is similar to the maximum absorption of conjugated dienes (200-300 nm).

(ii) Proton nmr of U1

The proton nmr of U1 was examined at 360 MHz on a Bruker spectrometer (10-15% solution in carbon tetrachloride). The results obtained suggest a structure in which both methyl groups are attached to olefinic carbon atoms:



δ ppm	Appearance	No. of Protons	Assignment
5.59	singlet	1	H_c
5.19	triplet	1	H_d (couples with x methylene)
2.00	multiplet	2	$(CH_2)_x$
1.98	apparent triplet	2	$(CH_2)_y$
1.71	doublet	3	(CH_3) A and B
1.67	multiplet	3	

The olefinic proton signals are clearly shown at 5.59 δ (H_c singlet) and at 5.19 δ (H_d triplet). The triplet appearance of this signal is explained by the fact that it couples with the methylene protons

$(CH_2)_x$. The other olefinic proton is not coupled. The methylene protons on either side of the double bonds, designated $(CH_2)_x$ and $(CH_2)_y$ appeared as a multiplet and a triplet at 2.00 δ and 1.98 δ respectively. The methyl protons attached to C-7 and C-9 appeared at 1.71 δ (doublet) and 1.67 δ (multiplet).

This spectrum confirms the proposal that this compound contains a conjugated diene system and because the branched methyl groups are involved in the diene bonds, they must be located at $\Delta 6, 8$ or $\Delta 7, 9$.

(e) Formation of maleic anhydride adduct

Diels-Alder reaction was carried out on U1 and on an authentic conjugated diene (18:2 9t, 11t). This latter was not entirely pure and contained some other 18:2 esters.

The product from the authentic diene was separated by tlc into two products. The less polar product, examined by glc, was found to be similar to the original impure diene except that the 18:2 (9t, 11t) ester had largely disappeared. The much more polar product was examined by mass spectrometry (mass spectrum 22, page 153). The spectrum was fairly complex with many fragments corresponding to $(CH_2)_nCOOCH_3$, C_nH_{2n-1}, C_nH_{2n-1}, or C_nH_{2n-3}. In addition there were fragments involving loss of one or more of CO, OCH_3, CO_2, $COOCH_3$, $CH_3(CH_2)_5$ and $(CH_2)_7COOCH_3$.

Treated in a similar way U1 failed to give an adduct but after exposure to light in the presence of iodine - a procedure known to promote configurational change - the ester gave a polar product which gave mass spectrum 23 (page 153) similar to that obtained

with the authentic diene. It was not possible to determine the full structure of this adduct and thus assign double bond positions but it is concluded that U1, though a conjugated diene, is not the trans, trans isomer.

(f) Double bond position by MS of hydroxylated derivative of U1

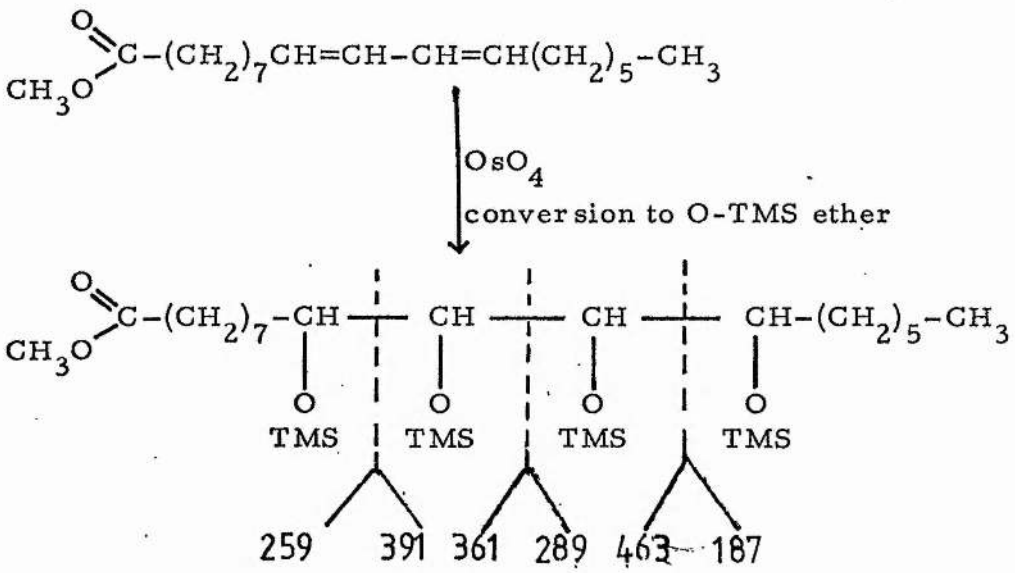
Mass spectrometry of methyl esters does not distinguish between positional unsaturated isomers. Several procedures have been suggested as a possible answer to this difficulty but none has proved to be entirely acceptable for the location of double bonds in polyene systems.

The transformation of polyene esters to polyhydroxy derivatives by treatment with osmium tetroxide (OsO_4) followed by mass spectrometry of the trimethylsilyloxy (O-TMS) derivative is nevertheless the most favoured method for the location of double bonds in polyunsaturated compounds. Fragmentation between vicinal CHOTMS groups is reported to produce large and diagnostic ions. The method has been applied to methylene-interrupted[144] and conjugated polyenes[145]. It has been reviewed and recommended by Minnikin[146].

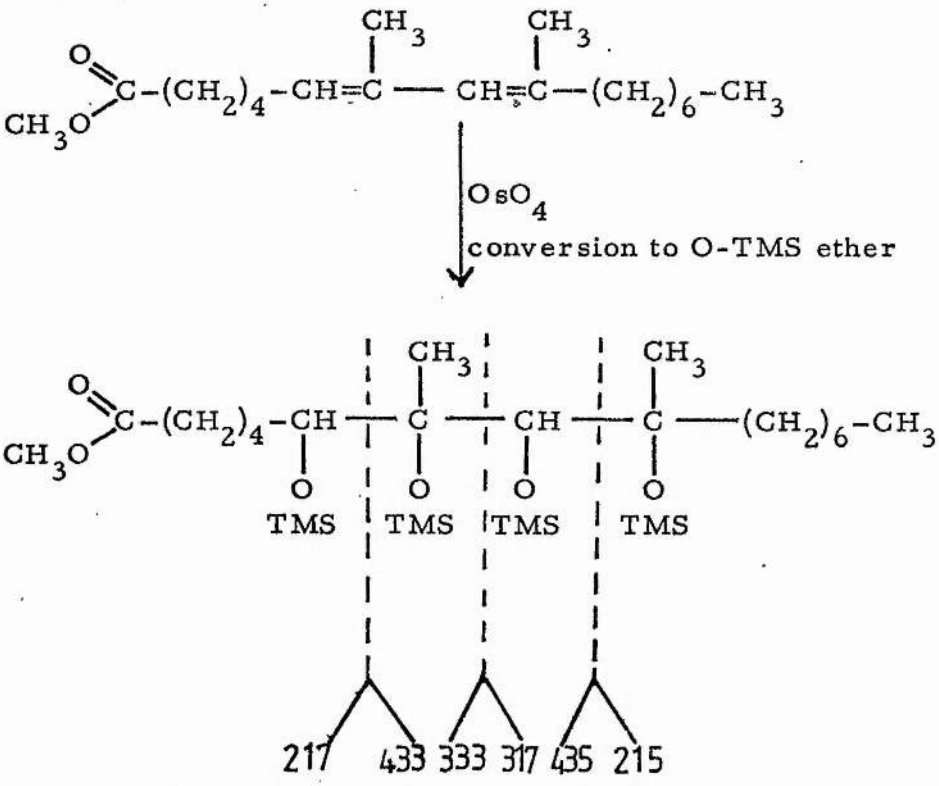
The U1 ester (88% purity) was transformed to the tetrahydroxy ester by treatment with OsO_4 and converted to the trimethylsilyloxy (O-TMS derivative). An authentic conjugated diene ester (18:2, 9t, 11t) was treated similarly. Both products were examined by mass spectrometry at 20 eV.

The mass spectrum 24 (page 154) of the O-TMS derivative of authentic 18:2 (9t, 11t) ester as expected did not display the molecular ion (654). The double bond positions are fixed by the

prominent peaks at m/e 187 and 259.



The mass spectrum 25 (page 154(a)) of the U1 O-TMS derivative has a base peak at m/e 215 and a significant peak at m/e 217. These two peaks unequivocally locate the double bonds at positions 6 and 8.



(g) Mass spectrometry of the pyrrolidide derivative of U1

After a comprehensive study of various oleic acid amides Andersson et al[63] found that the pyrrolidide gave a characteristic fragmentation pattern which indicated the position of the double bond in monounsaturated fatty acids[147]. The method has since been applied to methylene-interrupted polyenes[148-150].

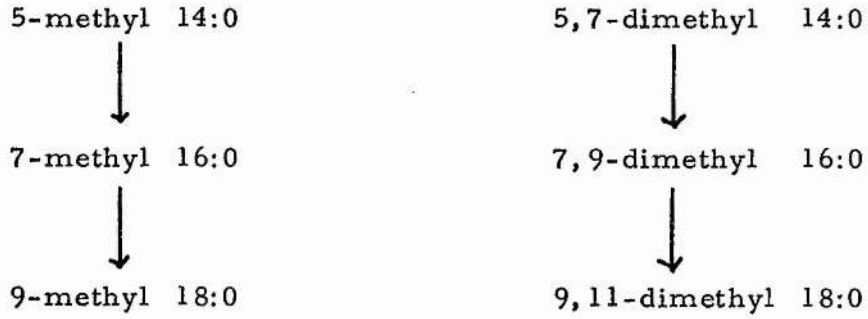
The mass spectrum 21 (page 152) of the pyrrolidide of U1 displayed a molecular ion of m/e 333 and a base peak at m/e 113 (the McLafferty rearrangement ion) indicating a C_{18} diunsaturated compound. The remainder of the spectrum was difficult to interpret and peaks at m/e 196, 208, 236 and 248 might suggest that the diene acid is Δ8, 10. This result is in conflict with that obtained from the tetrahydroxy TMS derivative. It is however uncertain how the result might be influenced by the fact that the branched methyl groups are each attached to olefinic carbon atoms.

2.4.4 Comments on the structure of branched-chain acids (other than those derived from phytol) in cod liver oil

From these studies of the branched-chain acids of cod liver ester it is concluded that unsaturated derivatives of 5-methyltetradecanoic, 5,7-dimethyltridecanoic, 5,7-dimethyltetradecanoic, 7-methylhexadecanoic, 7,9-dimethylhexadecanoic, 9-methyloctadecanoic, 7,9-dimethyloctadecanoic, and 9,11-dimethyloctadecanoic acids are present in trace amount. Among these, the 7,9-dimethylhexadecanoic acid predominates and it has been possible to show that this is probably present as a Δ6, 8 diene in a configuration other than trans, trans.

The location of methyl groups on alternate odd carbon atoms is unexpected, since involvement of propionate (or methylmalonate) in biosynthesis leads to branched methyl groups on even carbon atoms. Conjugated unsaturation is also unusual in animal lipids.

It is, however, noteworthy that the Δ6 and Δ7 derivatives of 7-methylhexadecenoic acid have been identified in fish lipids (see section 2.4.1). The carbon skeleton of these branched-chain acids (ignoring unsaturation) suggest that they belong to two families linked by chain-elongation, thus:



2.5 MONOENOIC ACIDS

2.5.1 Introduction

Monoene acids make up 40-60% of the total acids in most fish oils. They range in chain length from C_{14}-C_{24} with the C_{18} member(s) predominating (35-45%). Isomeric compounds differing only in the position of the double bond occur and these are difficult to separate from one another. The compounds seem to be almost wholly of cis configuration. Separation of monoene acids has been achieved by countercurrent distribution[95,151], silver ion chromatography[152], preparative glc[153-156], liquid-liquid chromatography[157,158], fractional distillation[159-162] and low temperature crystallisation[163].

Much work on the isolation and structural determination of monoethylenic unsaturation has been carried out on partially hydrogenated fish oils. Animals fed on diets rich in rapeseed oil and glyceryl trierucate show signs of cardiopathogenic changes[164-166], which have been attributed to erucic acid (cis-docos-13-enoic acid). Since hydrogenated fish oils are used in margarine manufacture, analysis and characterisation of the various isomers are important. Conacher et al[52] used preparative glc and silver ion tlc for the isolation and study of monoethylenic isomers in cardiac lipids of rats fed partially hydrogenated herring oil. They used ozonolysis to finally locate the double bonds. Conacher and Page[49] used a similar approach for studies on isomers of major monoenoic acids in rapeseed and partially hydrogenated rapeseed oil. Chromatographic determination of the monoethylenic geometric and positional isomers has been reviewed by Conacher[167].

The use of open tubular or capillary columns for the identification of isomeric monoethylenic fatty acids has increased since Lipsky et al[168,169] separated oleate from elaidate on a capillary column coated with ApL. Later Litchfield et al used DEGS[170] (diethyleneglycol succinate) and CNES[171] (α,β -cyanoethyl methyl siloxane polymer) coated columns for the separation of geometric isomers. Ackman and Hooper used capillary columns coated with BDS (butanediol succinate) and ApL phases to identify trans-hexadec-6-enoic acid in marine turtles[172], a sea anemone[173], and white jellyfish[139]. Using capillary column glc Ackman and McLachlan[80] recently examined in detail fatty acids of marine seaweeds. From a collaborative study on the determination of erucic acid in edible fats and oils, by capillary glc, Ackman et al[174] concluded that "the method of analysis under test appears to have been conveniently performed and to be sufficiently reproducible from one laboratory to another for the purposes for which it is intended". A close agreement between capillary glc and ozonolysis techniques has been reported[174]. Future trends in the isolation and identification of monoenoic fatty acids may involve combined use of HPLC and capillary glc. Recently Kirk et al[175] successfully used silver ion chromatography to separate erucic acid from other cis-docosenoic acid isomers in mixtures of rapeseed and herring oil fatty acids. Morris et al[176] had earlier separated isomeric cis and trans-octadecenoic acids by multiple development at low temperatures using tlc plates impregnated with silver nitrate.

2.5.2 Isolation

In this study preliminary fractionation of cod liver fatty acids was achieved by low temperature crystallisation. Further isolation and purification was carried out by argentation tlc and preparative glc. Fatty acids were used in place of methyl esters for the low temperature crystallisation because: (i) the relative solubility of fatty acids and their esters increases with higher unsaturation and decreases with longer chain length; (ii) unsaturated fatty acids with cis configuration are more soluble than their trans isomers, but with methyl esters, the solubilities of cis and trans are more alike; and (iii) the crystallisation of unsaturated esters is limited by their greater solubility compared to the corresponding acids.

Low-temperature crystallisation of the fatty acids from acetone was carried out at temperatures ranging from -15° to -75°, as shown in Scheme V. Five different fractions were obtained. The component fatty acids in the various fractions are detailed in Table 12. The composition of precipitates A-D and of the final mother liquor are set out in Table 12. The first precipitate (6%) is mainly saturated acids (89%) and contains only small amount of monoene acids (10%). The second precipitate (also small, ~ 7%) is mainly a mixture of saturated (34%) and monoene (42%) acids. The remaining precipitates are larger (18% and 26%) and contain monoenes as the major component (76%, 66%). The mother liquor (28%) is enriched in polyenes (72%) and contains only 21% of monoene acids. The C_{22} and C_{20} monoenes are concentrated in precipitate C whilst the C_{18} and C_{16} monoenes are found mainly in precipitate D.

Table 12. Separation of cod liver fatty acids from acetone solution by low temperature crystallisation: % composition of various fractions

Fractions	Total	ppt(A)	ppt(B)	ppt(C)	ppt(D)	ML(D)
Temp. of crystallisation (^{o}C)		-20	-40	-55	-75	
Weight (g)	(100)	6.4	4.3	17.7	25.6	28.0
Component esters (Wt,%)						
Assignment						
14:0	3.4	5.7	4.9	5.0	2.6	1.0
15:0	0.4	0.9	0.8	-	0.4	0.3
15:0 br/15:1	0.3	0.2	0.3	0.2	0.3	0.4
16:0	9.8	62.3	29.9	11.1	3.0	0.4
16:1/P_{20}	8.7	1.5	4.7	3.0	13.0	13.9
18:0	2.6	17.3	-	2.8	1.1	1.3
18:1	22.0	2.9	11.8	19.1	41.8	0.2
18:2 n-6	1.9	0.3	1.2	-	2.3	4.1
20:1/18:3 n-6	13.2	2.2	13.3	30.2	3.2	3.9
20:2/18:4 n-3	2.6	0.3	1.3	0.4	2.1	6.0
22:1/20:3 n-9	8.2	1.6	10.5	22.9	6.6	1.6
20:4 n-3	0.7	0.1	0.3	0.2	0.5	1.6
20:5 n-3	10.6	1.2	5.2	1.4	9.2	26.9
24:1	0.7	1.2	1.2	1.0	0.6	1.4
22:4 n-6	0.7	-	-	-	0.5	-
22:5 n-6	0.4	-	-	-	0.3	0.6
22:5 n-3	1.1	-	0.5	0.1	1.0	3.0
22:6 n-3	12.1	1.5	6.0	1.6	10.3	30.6
Others	0.6	0.8	8.1	1.0	1.1	2.8

Low temperature crystallisation carried out in this way failed to produce pure acids and further methods were therefore used to improve the purity of the monoene acids.

An attempt to separate precipitate C into its components by reversed-phase chromatography using 5% silicone fluid (DC2000/5CS) in diethyl ether according to the procedure described by Malin[86] and Mangold[88] was unsuccessful.

Silver ion tlc was therefore used to separate the monoene-rich precipitate C mainly according to number of double bonds. Esters (100 mg, ppt C/plate) were applied to plates covered with silica (0.5 mm) containing silver nitrate (10%) which were developed in PE7.5 . Separated bands were located by 2',7'-dichlorofluorescein (0.2%). The first band, a small narrow band of saturates, was followed by a broad band of monoenes, and by a narrow but strong band of polyenes. The recovered monoene band contained about 98% of monoenes (16:1, 5%; 18:1, 23%; 20:1, 42%; 22:1, 28%).

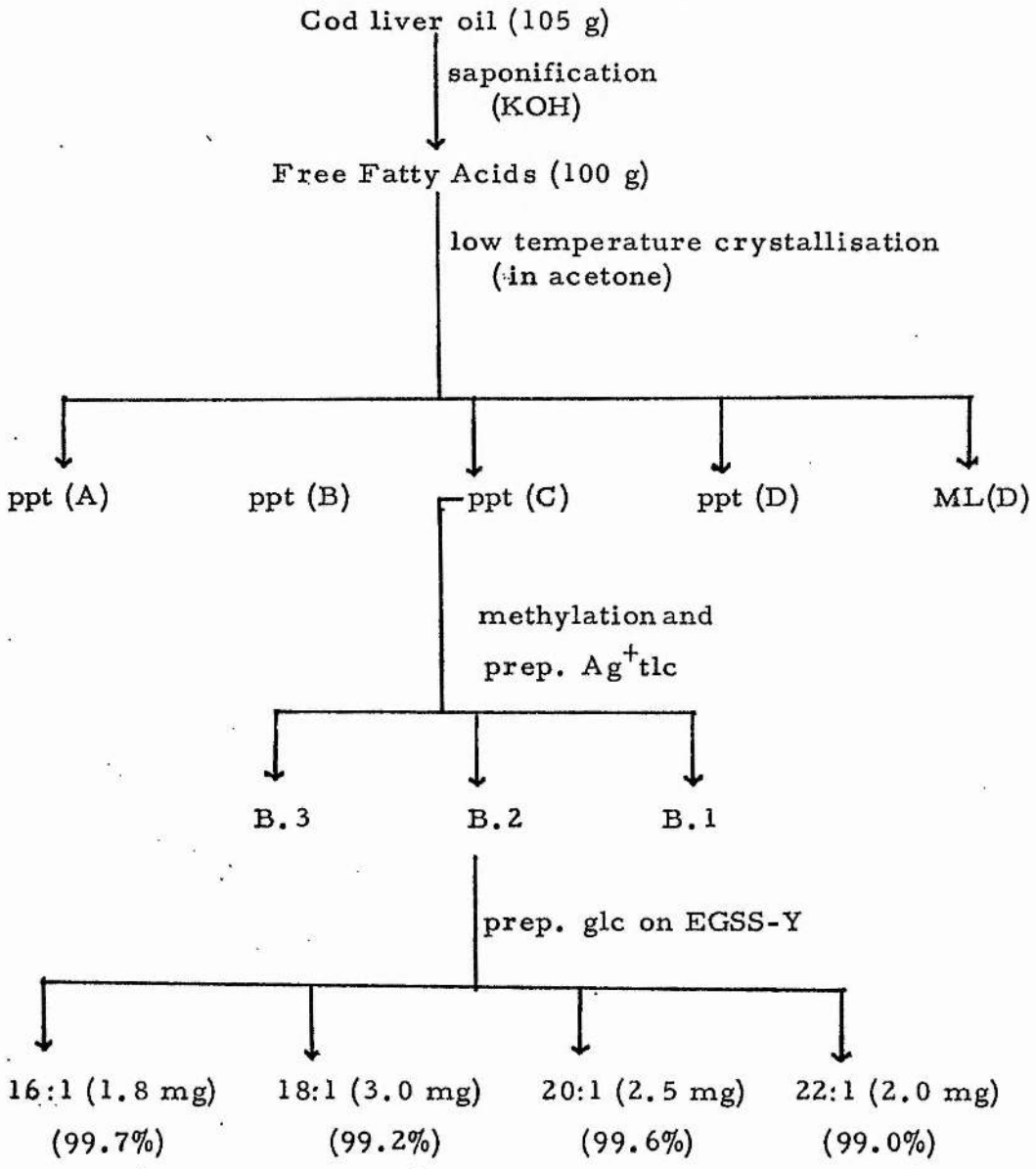
Attempts to isolate individual monoene esters by reversed-phase chromatography were not entirely successful and are reported only briefly. When the silica was silanised prior to preparation of the tlc plates according to the procedure of Howard and Martin[177] and of Litchfield[87] separation by chain-length was poor. More success followed with the Ord-Bamford[178] method of using dimethylchlorosilane or trimethylchlorosilane to produce a lipophilic surface. Monoenes of differing chain-length were separated (purity > 95%). The bands could not be made visual with either Rhodamine B or 2',7'-dichlorofluorescein but iodine provided the necessary staining of the lipid monoenes without reacting irreversibly with the monoene esters. This method was abandoned because of a number of other

difficulties associated with this procedure.

Preparative glc was therefore used to isolate individual monoethylenic esters in a form pure enough for chemical characterisation. Whilst it is known that some long-chain esters such as the conjugated trienoates and α-hydroxy conjugated dienoates undergo stereomutation and dehydration respectively[179,180], there is no evidence of any changes in monoene or non-conjugated polyene esters[181,182]. Nevertheless it is desirable to take the following precautions. The column temperature must be high enough to promote rapid elution but not so high as to lead to possible structural modification. The outlet collecting device was therefore only 5-10^{o} above the column temperature. The carrier gas flowed as rapidly as possible (consistent with good separation) to minimise the residence time of the esters on the column.

With these points in mind the monoene esters already concentrated by silver ion chromatography were separated according to chain-length on an EGSS-Y column at 200^{o} by preparative glc. Each isolated monoene ester fraction was recovered, weighed and analysed by the glc on SP2340. Over 99% purity of each ester was obtained. Further purity check on these isolated pure esters was carried out using 0.25 mm thickness tlc plates. The isolated fractions were applied to the tlc plates alongside authentic monoene materials and the original precipitate C obtained by low temperature crystallisation. Plates were developed in PE10 and bands were located by charring. Only a methyl ester band was obtained in each case, showing that the isolated compounds were methyl esters only. A flow diagram for the separation and analysis of the monoenoic acid esters is shown in Scheme V.

Scheme V. Flow diagram for the isolation of component monoenoic acids



Note: Isolated monoenes (7 99% purity) were checked by tlc and by glc on SP2340 column

2.5.3 Structural identification

For the structural identification of the positional isomers of the monoene esters oxidative cleavage of the double bonds and subsequent glc identification of the products was adopted, specifically ozonolysis yielding aldehydes and aldehydo-esters gave the best results.

Use of ozonolysis for the location of double bonds

The products obtained from ozonides by reaction with triphenylphosphine (TPP) are aldehydes (A) and aldehydo-esters (AE) and authentic samples of these are required for the identification of the oxidation products. Aldehydes are readily available but aldehydo-esters are not and must be prepared.

Authentic aldehydes and aldehydo-esters were obtained from pure (> 99%) samples of synthetic 18:1 esters having the double bond at various positions on the chain. Authentic methyl octadecenoates were individually ozonised and the resultant ozonides reduced with TPP. The aldehydes and the corresponding aldehydo-esters were analysed by the glc using ApL (5%) and SP2340 (10%). A series of aldehydes and aldehydo-esters were obtained from the Δ8 through Δ13 18:1 acids. Standards of authentic aldehydes analysed on ApL (5%) and SP2340 (10%) displayed retention times and ECL identical with those obtained by reductive ozonolysis of the synthetic esters.

(a) Analysis of ozonolysis products

Authentic aldehydes, aldehydo-esters and monobasic esters (C_6-C_{16}) were examined by temperature-programmed glc on ApL (5%) and SP2340 (10%) columns under the same conditions as the

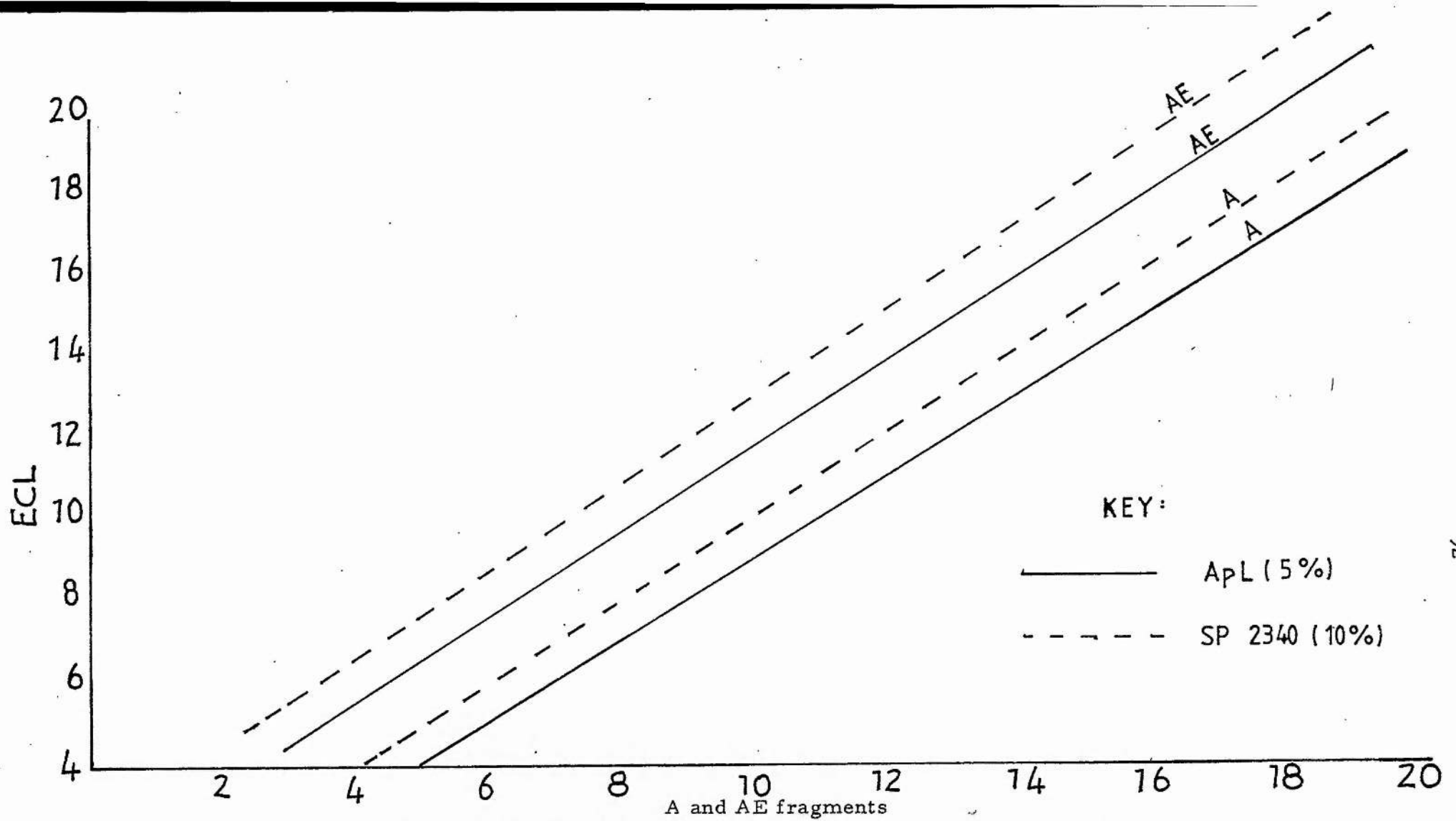


Fig. 1. Graph of ECL against chain lengths of aldehydes (A) or aldehydo-esters (AE) based on authentic methyl alkanoates.
Columns: ApL (5%); efficiency 700 theoretical plates/foot
SP2340 (10%); efficiency 1290 theoretical plates/foot
Temperature-programmed (80-200°C). After holding at 80° for 5 min the column temperature was raised to 200° at 4°/min and kept at that level until analysis was complete.

ozonolysis products. Equivalent chain lengths of the aldehydes and aldehydo-esters were expressed in terms of the retention behaviour of methyl alkanoates[183] and were calculated using the two ester standards closest in retention time to that of the peak under consideration. Graphs of ECL against chain length of the aldehydes and aldehydo-esters gave a straight line relationship (Fig. 1).

The ECL of the ozonolysis products (aldehydes and aldehydo-esters) were also calculated by reference to the C_6-C_{16} monobasic methyl esters, and identified by use of the log plot already obtained for the authentic compounds. On polar and non-polar columns under isothermal conditions some aldehydes overlapped with some aldehydo-esters. A temperature-programmed system (80-200°) was therefore used to separate the various aldehydes and aldehydo-esters. A trace of the temperature-programmed elution of the fragments is shown in Fig. 2.

(b) Calculation of weight % and conversion to mole %

Ozonolysis of a monoene ester should produce the expected fragments (aldehydes and aldehydo-esters) in equimolar amounts. However, the FID does not respond equally to equimolar amounts of aldehydes and aldehydo-esters of different chain lengths. Because of this difficulty the aldehydo-ester fragments alone are usually used to quantitate the relative amounts of monoenoic esters[184,185].

A calculation procedure suggested by Dal Nogare and Juvet[186] which takes cognisance of the fact that the FID response does not allow for carbon atoms attached to oxygen, was therefore used in

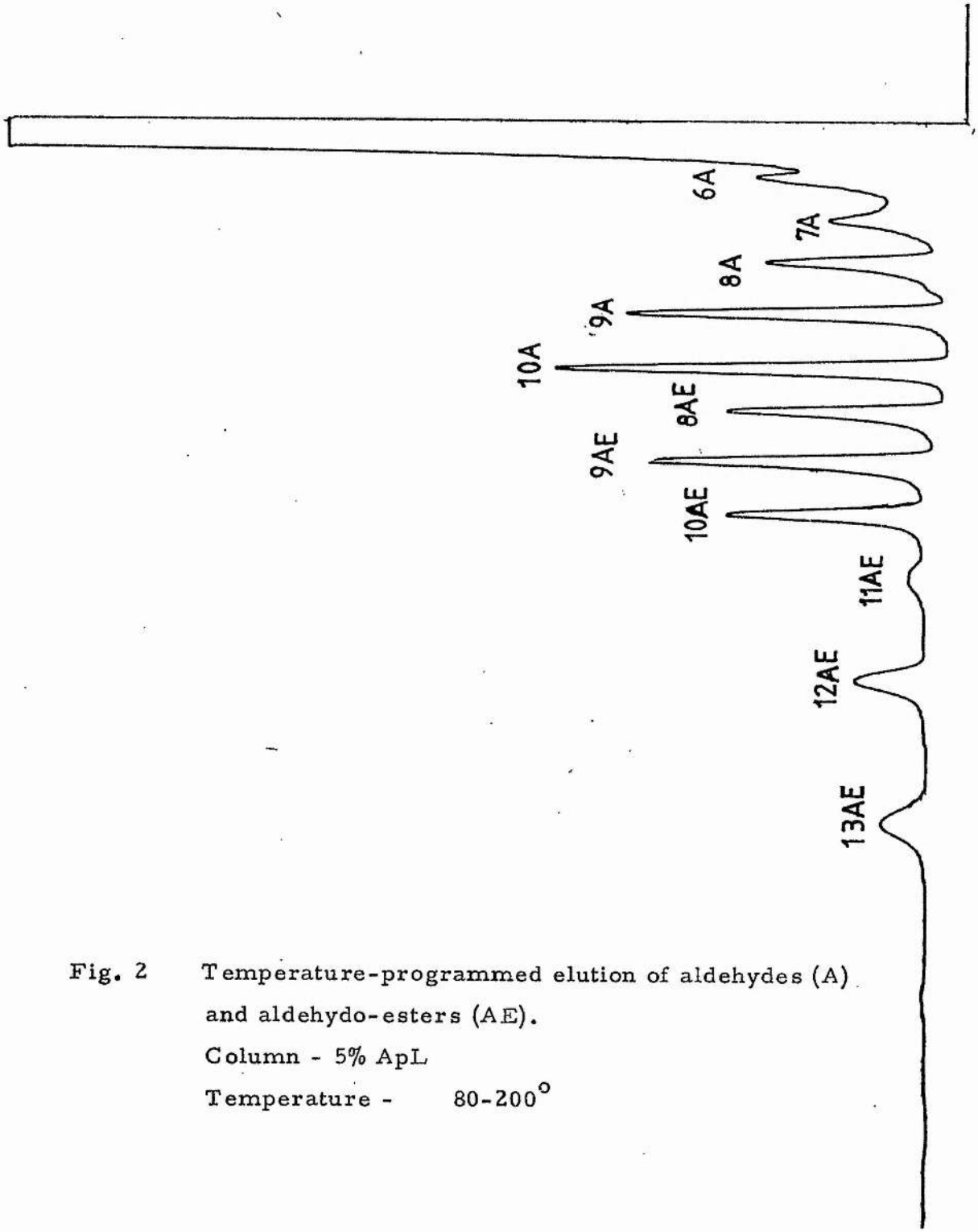


Fig. 2 Temperature-programmed elution of aldehydes (A) and aldehydo-esters (AE).
Column - 5% ApL
Temperature - 80-200°

in this study.

$$\text{Molar units for each peak} = \frac{\text{ht} \times \text{'w'}}{12(n-f)}$$

where ht = height of peaks of fragments, 'w' is the width of the peak at half height, n is the number of carbon atoms in the aldehyde or aldehydo-ester and f is 1 for aldehydes and 2 for the aldehydo-esters.

Mole percentages were calculated by normalising the results of the aldehydes and the aldehydo-esters separately. The C_{16}, C_{18}, C_{20} and C_{22} monoene esters isolated by preparative glc were analysed in this way.

Octadecenoates

double bond position	Δ8	Δ9	Δ10	Δ11	Δ13
AE (mole %)	1.8	65.6	2.0	29.1	1.5
A (mole %)	0.7	69.3	1.2	27.3	1.5
Mean (mole %)	1.0	68.0	2.0	28.0	1.0

Eicosenoates

double bond position	Δ9	Δ10	Δ11	Δ12	Δ13
AE (mole %)	6.3	0.6	91.5	0.4	1.2
A (mole %)	6.0	0.4	91.6	trace	1.0
Mean (mole %)	6.0	1.0	92.0	trace	1.0

Docosenoates

double bond position	Δ10	Δ11	Δ12	Δ13
AE (mole %)	5.0	90.7	1.4	2.9
A (mole %)	2.5	87.4	1.6	8.5
Mean (mole %)	4.0	90.0	1.0	5.0

Hexadecenoates

double bond position	Δ5	Δ7	Δ8	Δ9	Δ10	Δ11
AE (mole %)	10.0	2.0	3.0	47.0	23.0	15.0

In this sample (16:1) the short chain aldehydes were recovered only in small amounts, loss probably occurring during the concentration procedure. The aldehydes were not therefore quantitatively determined but qualitatively they correspond to their respective aldehydo-ester counterparts. The double bond locations were therefore assigned based on the aldehydo-esters only.

Table 13. Summary: Double bond positions (by reductive ozonolysis)

	- Δ (Mole %)					
	8	9	10	11	12	13
16:1 (99.7%)	3	47	23	15	-	-
18:1 (99.2%)	1	68	2	28	-	1
20:1 (99.6%)	-	6	1	92	-	1
22:1 (99.0%)	-	-	4	90	1	5

[Note: The 16:1 also had double bonds at Δ5 (10 mole %), and Δ7 (2 mole %)]

2.5.4 Comments on the biosynthesis of the monoenoic acids

The present results summarised in Table 14, show the presence of three n-7 acids predominantly C_{18}, three n-9 acids which are mainly C_{18} and C_{20} and two n-11 acids mainly C_{22}. The n-9 acids are probably produced from stearate and oleate

thus:

$$18:0 \longrightarrow 18:1(9) \longrightarrow 20:1\ (11) \longrightarrow 22:1\ (13)$$

In this sample of cod liver oil the C_{18} and C_{20} members are the most significant. The n-11 monoene acids (mainly C_{22}) are probably obtained from 20:0 and the n-7 acids (mainly C_{18}) from 16:0 thus:

$$20:0 \longrightarrow 20:1\ (9) \longrightarrow 22:1\ (11)$$

$$16:0 \longrightarrow 16:1\ (9) \longrightarrow 18:1\ (11) \longrightarrow 20:1\ (13)$$

The C_{16} monoene fraction contains the n-10 acid in addition to n-7 isomer. Along with these major monoene acids 14:1, 15:1, 17:1, 19:1 and 24:1 acids were tentatively identified as minor components.

Table 14. Major monoene acids (% of total acids) present in cod liver oil

Chain length	Total acids	n-11	n-9	n-7
C_{16}	1.5	-	-	47
C_{18}	22.4	-	58	28
C_{20}	13.8	6	92	13
C_{22}	8.6	90	5	-

2.5.5 General remarks on the ozonisation procedure

In order to minimise side reactions/contaminants during the ozonisation of the monoene esters the following precautions were adopted:

(a) The solution of methyl ester in methylene chloride was made as dilute as possible.

(b) The temperature of the ester/methylene chloride solution was maintained at -70 to -75° throughout the ozonisation procedure.

Direct bubbling of O_2/O_3 mixture into pre-cooled ester/methylene chloride (-75°) did not produce side reactions. Addition of ester after ozone had been bubbled into methylene chloride cooled to -70°, had no advantage over the former method.

(c) Excess ozone was driven off quickly from the ozonide mixture with a gentle stream of nitrogen before reduction with TPP.

(d) Reduction with TPP was carried out in dilute solution (methylene chloride) of ozonides at low temperature preferably at -70°.

(e) During concentration of the reduction products care was taken not to volatilise the short-chain aldehydes. Glc analysis of the reduction products was performed soon after concentration. This is especially important if quantitative analysis is required.

(f) 20 mg of TPP was found adequate for complete reduction of 1 mg ester samples. Direct addition of TPP crystals into the reaction vessel was as effective as addition as a solution of methylene chloride.

Despite its accuracy in the location of double bonds and the obvious advantages over the potassium permanganate-periodate technique, the technique had the following disadvantages.

(a) Although triphenylphosphine oxide elutes late after the aldehydes and aldehydo-esters, it has a deteriorating effect on the column performance. Incomplete removal of the triphenylphosphine oxide from the column resulted in poor separation. Consequently the number of runs each day was restricted and the column was purged overnight. Injection of HMDS was found helpful in deactivating the column. With excess TPP ($>$ 20 mg/1 mg

sample), both the oxidised and unreacted TPP appear on the glc trace, with the latter eluting before the former. Both, however, elute a long way after the reduction products.

(b) Short-chain fragments such as pentanal down to propanal did not appear on the glc under the temperature programmes used and the column (5% ApL) could not be satisfactorily operated below 80°.

(c) Some reduction products which overlapped on the ApL column were separated on SP2340.

On the other hand the technique showed no side reactions, the ozonolysis products were mainly aldehydic in structure, the procedure is quick and does not require transfer of sample and the products are readily analysed by standard glc technique.

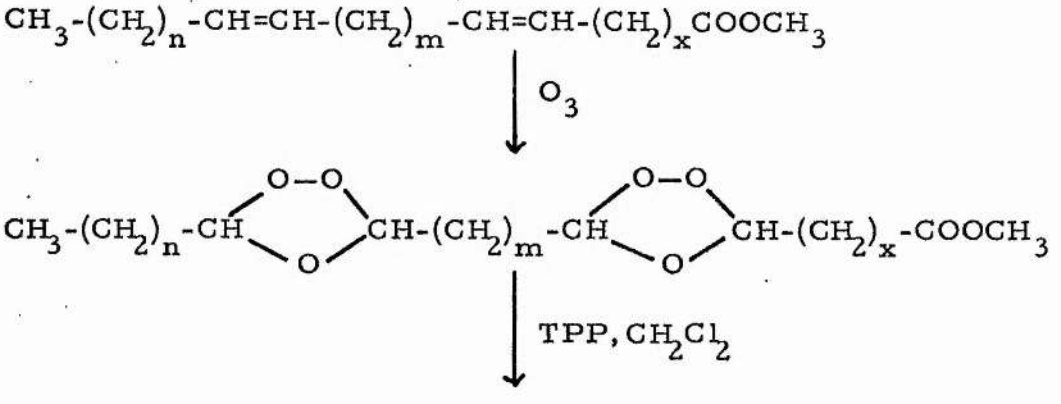
2.6 DIENE FATTY ACIDS

2.6.1 Introduction

The structural identification of a polyene acid is sometimes more difficult than that of a monoene acid and the identification of isomeric mixtures of polyene acids is even more difficult.

However, several techniques have been used with partial success to solve this problem. Scholfield et al[187] used capillary glc for the identification and determination of dienoic isomers. In this regard a fair knowledge of the sample under investigation and availability of suitable standards are pre-requisites for correct identification.

The most commonly used chemical procedure is ozonolysis. Stein and Nicolaides[188] and Kleiman et al[183] adopted ozonolysis followed by reduction with triphenylphosphine thus:



$CH_3(CH_2)_nCHO$ + $OHC-(CH_2)_m-CHO$ + $OHC-(CH_2)_x-COOCH_3$

(aldehyde) (dialdehyde) (aldehydo-ester)

This is subject to the following difficulties. (i) The dialdehyde fragments are not readily identified by glc. Pappas et al[189] suggested that the dialdehyde fragments are subject to polymerisation

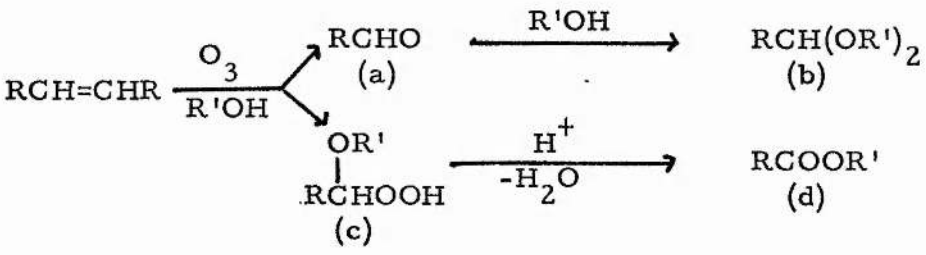
and may for this and other reasons give reduced yields. (ii) Though more stable than the aldehyde or dialdehyde fragments, the aldehydo-esters are sometimes difficult to identify because of the problem of securing authentic standards. (iii) Quantitative problems also arise with this method due to differences in volatility (aldehydes), column loss and differential detector sensitivity of the several breakdown products[190,191].

Many modifications of the ozonolysis procedure have been adopted. Johnson et al[192] reduced the ozonides with sodium borohydride giving alcohols, alcohol ester and diol fragments. Using a microozonisation apparatus and a suitable glc column they readily identified the alcohols and alcohol ester fragments.

Sebedio and Ackman[193] carried out the ozonolysis reaction of methyl linoleate in BF_3-MeOH solution, extracted the ester products with methyl cyclohexane and analysed them by isothermal glc on an open-tubular wall-coated column. They isolated and identified methyl hexanoate and dimethyl nonanedioate but failed to identify dimethyl malonate. They reported the decomposition of dimethyl malonate during oxidative decomposition of the ozonide. Dimethyl malonate has also been shown to be susceptible to decomposition during glc and has a relatively low response in the FID detector[194].

For non-methylene-interrupted octadecadienoic acid, Ratnayake and Ackman[195] modified the use of BF_3-MeOH. They first reduced the carboxyl group of the acid to an alcohol and ozonised the alcohol in BF_3-MeOH thus splitting the ethylenic bonds to methyl esters. As in the previous methods already described the C_3 fragment was not detected in this procedure. Neumeister

et al[196] described another ozonolysis procedure which furnishes esters directly. They carried out the ozonolysis in alcohol as solvent in the presence of anhydrous hydrogen chloride. Esters (d) are produced via aldehydes (a) and acetals (b) and/or via alkoxyhydroperoxides (c).



Hitherto, none of the methods discussed so far has satisfied the criteria set by the official AOCS standard technique and a satisfactory method for the location of double bonds of PUFA is still sought. There follows in this project an account of the isolation of some diene acids from cod liver oil and of attempts to identify these.

2.6.2 Isolation

In this study dienoic fatty acid esters were isolated from cod liver oil by a combination of low temperature crystallisation, preparative silver ion tlc and preparative glc. The mother liquor 'C' obtained by low temperature crystallisation (section 2.5) was used as an enriched source of dienoic esters. It was methylated in the usual manner and methyl esters were applied to tlc plates (0.5 mm thickness, 200 mg/plate prewashed in PE10). The plates were then developed in petrol-diethyl ether-acetic acid (80:20:1) and the purified methyl esters, extracted with 10%

methanol in diethyl ether, were reapplied to silver ion tlc plates (0.5 mm thickness, 150mg/plate) and developed in PE10 separating the esters into four distinct bands. These were mainly saturated (band 1), monoene (band 2), diene (band 3) and polyene esters (band 4).

The combined bands 3 and 4 were reapplied on silver ion plates (0.5 mm thickness), and developed first in PE20 and then PE40. The diene band moved far ahead of other polyenes. Throughout this silver ion chromatography authentic diene (18:2 w6) was also applied to the plate to aid in the location of the diene band. The composition of the diene band after the first and second silver ion separations is shown in Table 15. It contained 18:2 (ca 70%), 16:2 (ca 17%), and 20:2 (ca 11%) with traces of 14:2 and 22:2 .

Table 15. Component acids (%, wt) of the diene bands (a) (X1 Ag^+tlc) and (b) (X2 Ag^+tlc) on SP2340, 185°

Assignment	band (a)	band (b)
14:0	trace	trace
14:1	0.4	0.5
14:2	0.1	0.1
16:1	1.4	1.4
16:2	12.2	16.6
16:3	2.2	-
17:2/18:3?	-	2.7
18:2	61.5	67.0
18:3	5.5	-
18:4	2.3	-
20:2	10.1	10.6
22:0	1.7	-
22:1	1.4	-
22:2	0.9	1.2
24:0	0.1	-
24:1	0.2	-

Preparative glc

Preparative glc was used to obtain the various diene fatty acid esters in pure form. This was carried out as previously described on a 20% EGSS-Y column operated at 200°. The C_{16}, C_{18} and C_{20} dienes were isolated with a purity > 99.0%.

2.6.3 Structural identification

Structural identification of the various dienoic esters was undertaken using hydrogenation to confirm chain length and ozonolysis to locate double bond positions. Hydrogenation of the diene esters was carried out in the presence of 10% palladium on charcoal. The resulting saturated esters were analysed on both 2.5% ApL and 10% SP2340 columns to establish the chain length of each product. Authentic dienoic esters hydrogenated and analysed on the same columns as the test samples gave similar results.

(a) Ozonolysis procedure

The individual diene fatty acid esters isolated from cod liver oil (> 99% purity) were separately ozonised in ethanol and the ozonides reduced by sodium borohydride. The fragments, alcohol, alcohol ester and diol were identified by gas chromatography in comparison with authentic samples of each type of product. Because alcohol ester standards are not readily available, the expected alcohol and alcohol ester fragments were prepared from authentic mono- and dienoic fatty acid esters.

Preparation of alcohol and alcohol ester fragments

The authentic monoenoic and dienoic esters (≥ 99% purity) listed below were ozonised in ethanol and the ozonides were reduced to alcohols, alcohol esters and C_3 diols. These products were analysed by temperature-programmed glc (40-240°C) on a 1:14 mixture of 10% OV-17 and 10% OV-225.

Unsaturated esters	Products		
	Alcohol	Hydroxy ester	Diol
18:1 Δ13	C_5	C_{13}	-
18:1 Δ11	C_7	C_{11}	-
18:1 Δ9	C_9	C_9	-
18:2 Δ9, 12	C_6	C_9	C_3
18:3 Δ6, 9, 12	C_6	C_6	C_3 (2 moles)
20:3 Δ8, 11, 14	C_6	C_8	C_3 (2 moles)

Monobasic esters (C_6-C_{16}) were temperature-programmed on the same column and the equivalent chain lengths of the various alcohols and alcohol esters were calculated (Fig. 3).

Alcohols	ECL	Hydroxy esters	ECL	Diols $HO(CH_2)_nOH$	ECL
C_5	5.4				
C_6	6.5	C_6	11.4	n=3	8.2
C_7	7.5	C_8	13.5	n=4	9.5
C_8	8.2	C_9	14.5	n=5	10.6
C_9	9.5	C_{11}	16.5		
C_{10}	10.5	C_{13}	18.4		
C_{12}	12.5				

These values indicate that there is a danger of overlap between the alcohol with n carbon atoms, the ester of the hydroxy acid with n-5

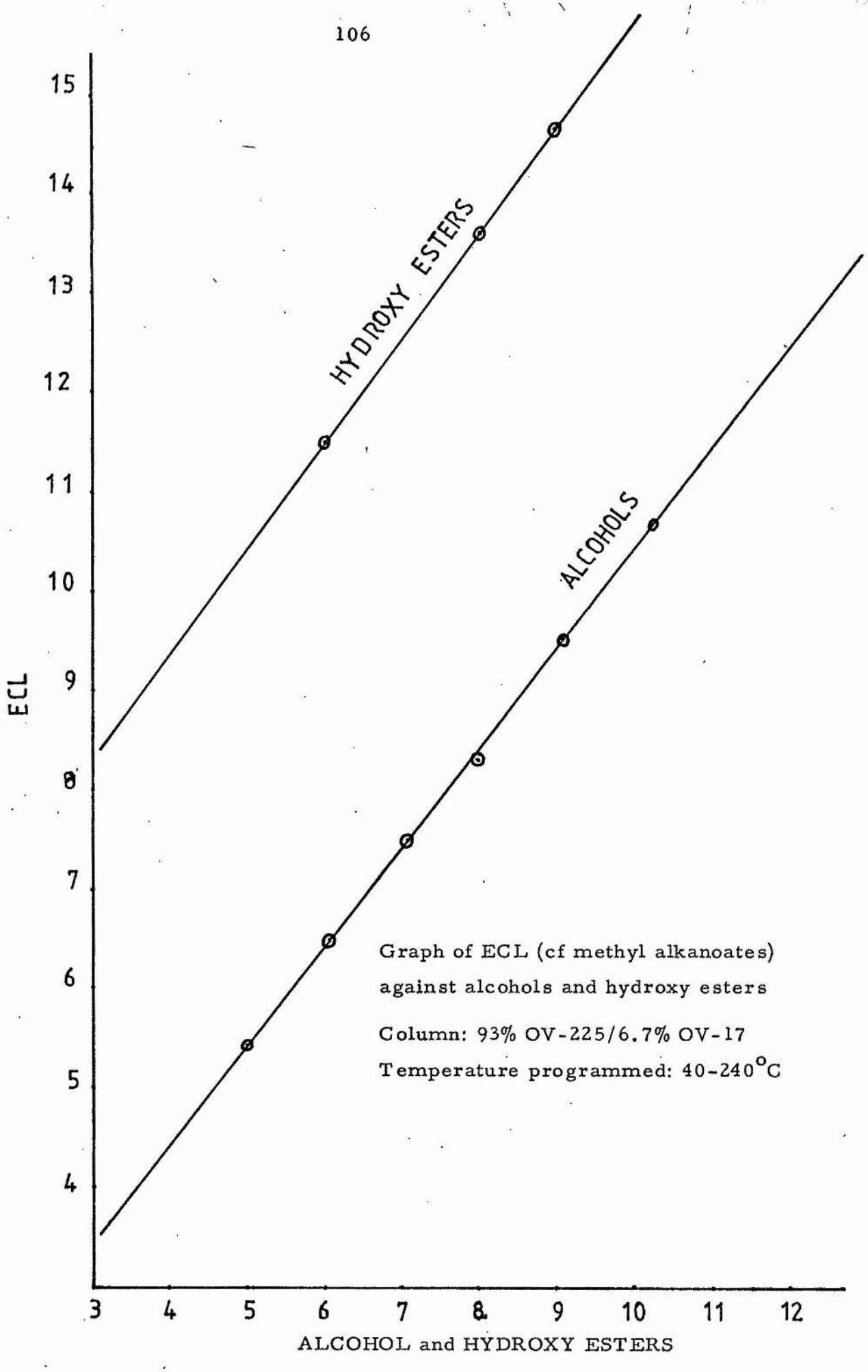

15
14
13
12
11
10
9
8
7
6
5
4
ECL
HYDROXY ESTERS
ALCOHOLS
3
4
5
6
7
8
9
10
11
12
ALCOHOL and HYDROXY ESTERS
Graph of ECL (cf methyl alkanoates)
against alcohols and hydroxy esters
Column: 93% OV-225/6.7% OV-17
Temperature programmed: 40-240°C

Fig. 3

carbon atoms and the diol with n-5 carbon atoms.

(b) Analytical results

The identification of the various ozonolysis products (alcohols and hydroxy esters) was based on the relative elution temperature (see section 2.5). In this study an internal standard was not used. Instead monobasic fatty acid esters (C_6-C_{16}) were used as explained in section 2.5 . With this method consistent ECL were obtained provided identical temperature programmed run was carried out on all samples and standards.

The height of each peak multiplied by the width at half height was used to calculate the mole percentage of the various fragments. The area percentages of the various fragments were converted to mole percentages by the procedure used in section 2.5 .

The following results were obtained from the dienoic acids isolated from cod liver oil.

Hexadecadienoate

C_4 alcohol	64 mole %
C_5 alcohol	6 mole %
C_9 hydroxy esters	30 mole %
double bond positions	Δ9, 12

Octadecadienoate

C_5 alcohol	5 mole %
C_6 alcohol	58 mole %
C_9 hydroxy ester	37 mole %
double bond positions at	Δ9, 12 (mainly)

Docosadienoate

C_5 alcohol	–	19 mole %
C_7 alcohol		81 mole %
C_{10} C_{12} hydroxy esters	]	present in small amounts - for reasons that could not be explained
double bond positions		major, Δ10,13 minor, Δ12,15 and possibly Δ10,15

These results for the reduction products of the diene acids were not satisfactory in terms of mole % obtained for both the alcohols and the hydroxy ester fragments. The mole % of the alcohols should be equal to those of the hydroxy esters. This was not the case in the above results. The results obtained for the docosadienoate are unexpected and no reasonable explanation is presently offered.

2.6.4 Advantages and disadvantages of the method

(a) Advantages

(i) Under alkaline conditions decomposition of ozonides to aldehydes is favoured and sodium borohydride reduces them to alcohol fragments in good yields. Consequently quantitative estimation of the alcohol and hydroxy ester fragments are obtained, because aldehydes and hydroperoxides as formed by the Criegee mechanism[46] are completely reduced to alcohol by sodium borohydride.

(ii) Sharp peaks with good base lines are obtained from the products including the shorter-chain alcohols.

(iii) The reduction procedure is fast, sensitive and reproducible and without interfering substances. Because of this, column

poisoning such as occurs when TPP is used as a reducing agent does not occur.

(iv) FID response to alcohols and hydroxy esters is good and the use of C factor to convert to mole % may not be necessary.

(b) Disadvantages

(i) Quantitative recovery of 1, 3-diol is not obtained.

(ii) Short-chain alcohols are prone to loss due to their high volatility.

(iii) The glc column must be operated from 40° in order to identify shorter-chain alcohols. Under this condition many columns would be unsuitable.

2.7 OTHER POLYENE ACIDS

2.7.1 Introduction

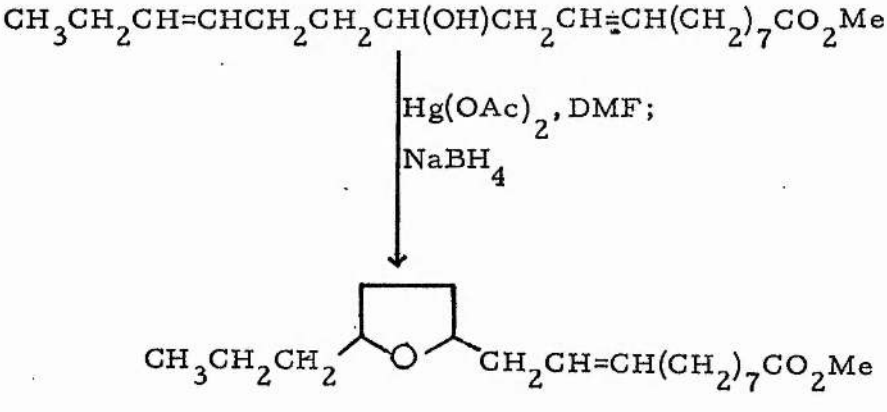
The oxymercuration reaction has been used for separating saturated from unsaturated fatty acids. Alkenes react with mercury salts in the presence of appropriate nucleophiles (solvents) to give a mercury derivative which can then undergo three major reactions.

(i) Stereospecific reversal of the addition reaction in the presence of halogen acid usually hydrochloric.

(ii) Replacement of the mercury-containing substituent by reaction with halogen.

(iii) Replacement of the mercury-containing substituent by hydrogen through reaction with sodium borohydride.

It has been shown that a hydroxyl group, present in an appropriate position with respect to the double bond, can participate in the oxymercuration in an intramolecular fashion to furnish cyclic ethers[197].



In the above example, methyl densipolate (12OH, 18:2 9c15c) undergoes oxymercuration-demercuration in dimethylformamide to furnish a 1,4-epoxide. In the extension of their study Gunstone and Inglis[198] observed that alken-1-ols with Δ3t, Δ4 or Δ5 unsaturation also undergo intramolecular oxymercuration to give

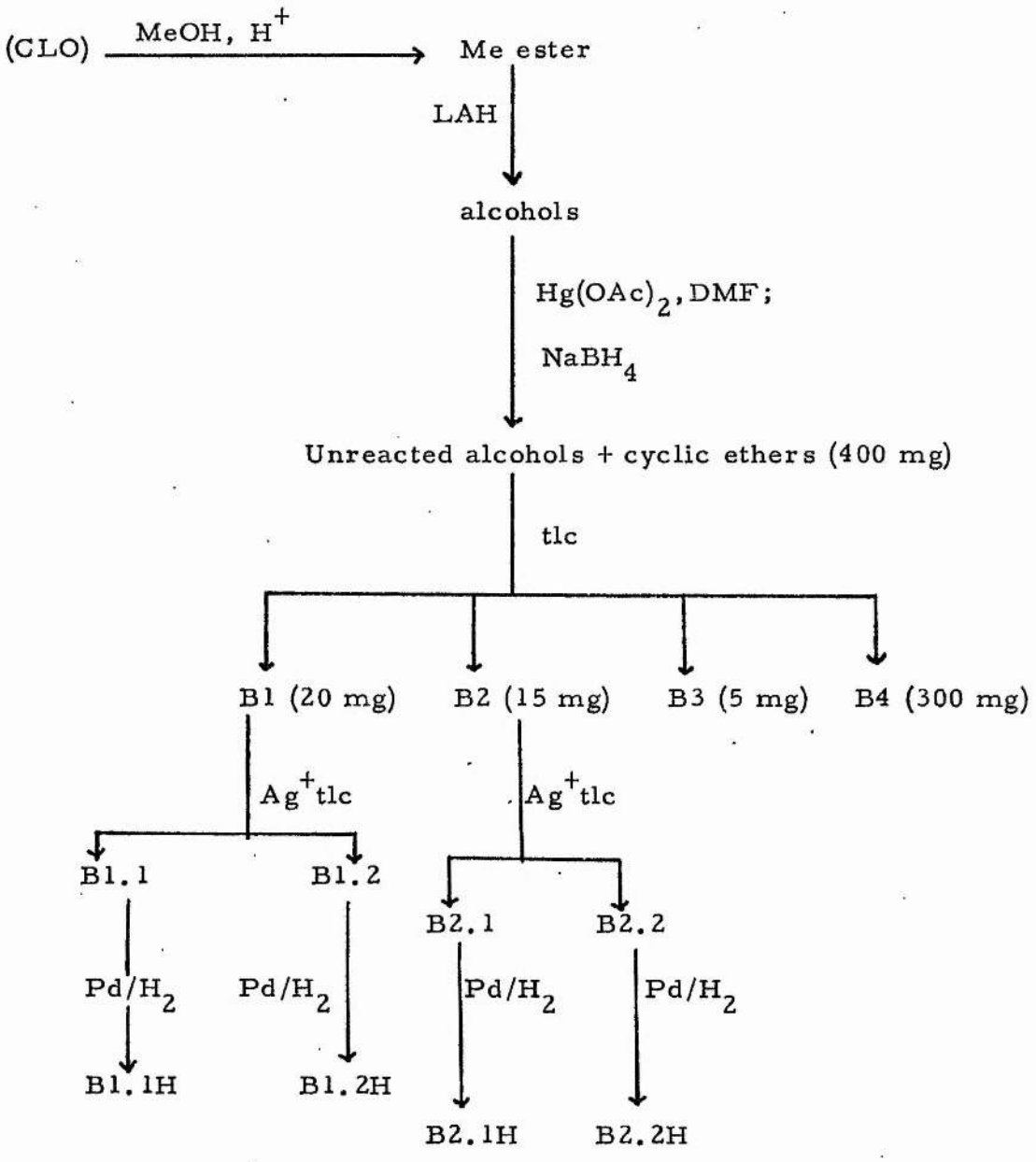
1,4- (from Δ3t and Δ4 alken-1-ols) or 1,5-epoxides (from Δ5 alken-1-ols).

In the present study of polyenes in cod liver oil, oxymercuration-demercuration was used to identify polenes having Δ3t, Δ4 or Δ5 unsaturation.

2.7.2 Isolation of polyenes

Mixed alcohols (ca 450 mg) from cod liver oil were treated with mercuric acetate in dimethylformamide (4 days) followed by demercuration with sodium borohydride. Unreacted alcohols and cyclic ethers (400 mg) were separated from each other by preparative tlc (Scheme VI).

Scheme VI. Isolation of polyene acids (having Δ3t, Δ4 or Δ5 unsaturation) from cod liver oil via oxymercuration-demercuration



Band 1 (fastest moving band) contained a cyclic ether of ECL 20.0 (92%) and another of ECL 18.8 (3.4%). Band 2 contained a cyclic ether of ECL 23.6 (98%), while band 3 contained a mixture of components with an ether of ECL 26.0 (58%) predominating. Band 4 (87% of the total) remained close to point of application and was unreacted alcohols. An aliquot of band 4 was analysed as alcohols and trimethylsilyl derivatives but further characterisation was not undertaken since these alcohols do not possess unsaturation at Δ3t, Δ4 or Δ5 positions. The small band 3 of uncertain mixture was not examined further.

Bands 1 and 2 were each further separated by preparative silver ion tlc (0.25 mm thickness in PE55) into two bands and each was hydrogenated with palladium charcoal (10%) as catalyst. The ECL and % composition of these are given in Tables 16 and 17.

Table 16. ECL and % composition of band B1 and the subfractions obtained from it (SP2340, 194°)

B1		B1 Ag^+		B1.2 Ag^+		B1.1 Ag H		B1.2 Ag^+H	
ECL	%	ECL	%	ECL	%	ECL	%	ECL	%
12.8	1.0	14.3	0.4	12.6	Tr	15.7	0.4	12.7	0.2
14.8	0.4	15.8	0.5	14.5	Tr	16.8	2.3	14.2	0.5
16.8	0.6	16.2	2.2	18.0	2.3	17.6	8.3	15.0	1.9
17.8	2.6	16.8	1.9	19.0	0.9	18.3	21.2	16.0	0.5
18.8	3.4	17.0	1.9	20.0	96.8	19.0	1.6	16.8	97.0
20.0	92.0	18.3	9.9			19.5	35.9		
		18.5	3.0			20.4	1.2		
		19.0	37.4			21.4	19.0		
		20.0	34.6			22.1	10.0		
		20.3	8.2						

Two authentic esters (18:1, Δ4 and 20:4, Δ5,8,11,14) were each carried through the same procedure of oxymercuration-

demercuration as the test sample. The ECL of these standards, as alcohols, TMS derivatives, and cyclic ethers are given in

Table 17. ECL and % composition of bands B.2 and subfractions obtained from it (SP2340, 194°)

B2		B2.1 Ag^+		B2.2 Ag^+		B2.1 Ag^+H^*		B2.2 Ag^+H^*	
ECL	%	ECL	%	ECL	%	ECL	%	ECL	%
11.8	0.1	12.2	2.2	20.0	4.0	13.8	0.7	16.8	9.6
13.2	0.3	12.6	2.6	23.6	96.0	16.4	2.7	18.2	10.0
15.8	0.4	13.6	7.8			17.6	11.0	19.8	80.4
17.4	0.8	14.6	1.6			18.3	20.4		
19.0	0.9	15.8	6.4			19.5	44.3		
23.6	97.5	16.2	4.3			21.4	16.9		
		16.7	3.3			23.4	4.0		
		17.2	2.3						
		17.6	30.3						
		18.6	22.1						
		19.0	5.2						
		20.0	6.9						
		21.0	2.8						
		22.0	2.2						

* Ag^+H = hydrogenated sample

table 18. Included in this table also are the results of Gunstone and Inglis for comparison.

The isolated cyclic ethers were finally examined by mass spectrometry.

2.7.3 Results and Discussion

The major polyene acids present in cod liver oil belong to the n-3 series or the n-6 series with the former predominating. The

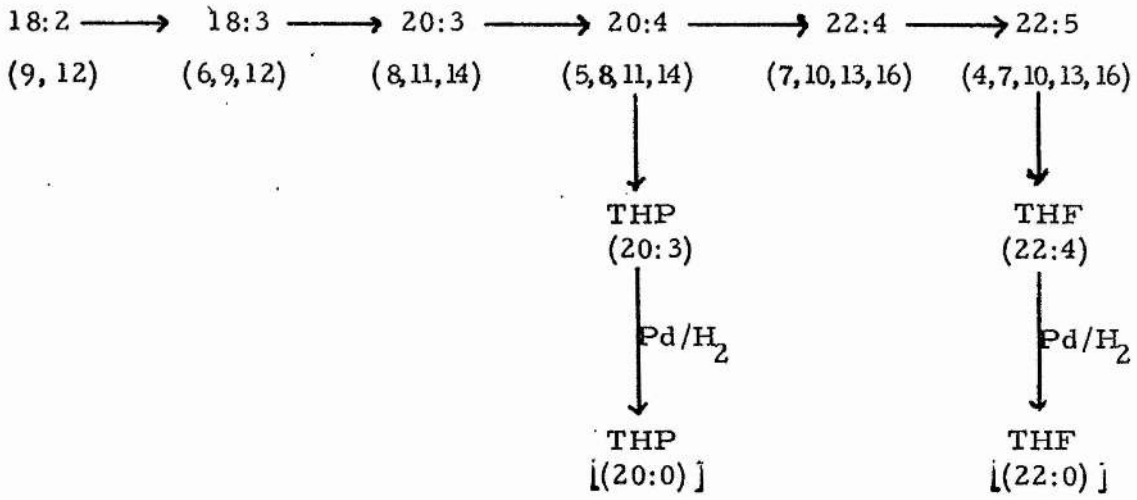
Table 18 ECL of 18:1 (4), and 20:4 (5, 8, 11, 14) as alcohols, TMS derivatives and cyclic ethers on SP2340 and some earlier results[a]

	RCO_2Me 194°	RCH_2OH (194°)	RCH_2OSiMe_3 (172°)	Cyclic ethers (194°)	Hydrogenated/ ether
18:1 (Δ4)		22.1		15.2 16.1[a]	
20:4 (n-6)	22.6	26.1	17.8	18.8 18.6[a]	16.8 17.0[a]
20:5 (n-3)				19.4[a]	17.0[a]
22:6 (n-3)				22.6[a]	19.0[a]

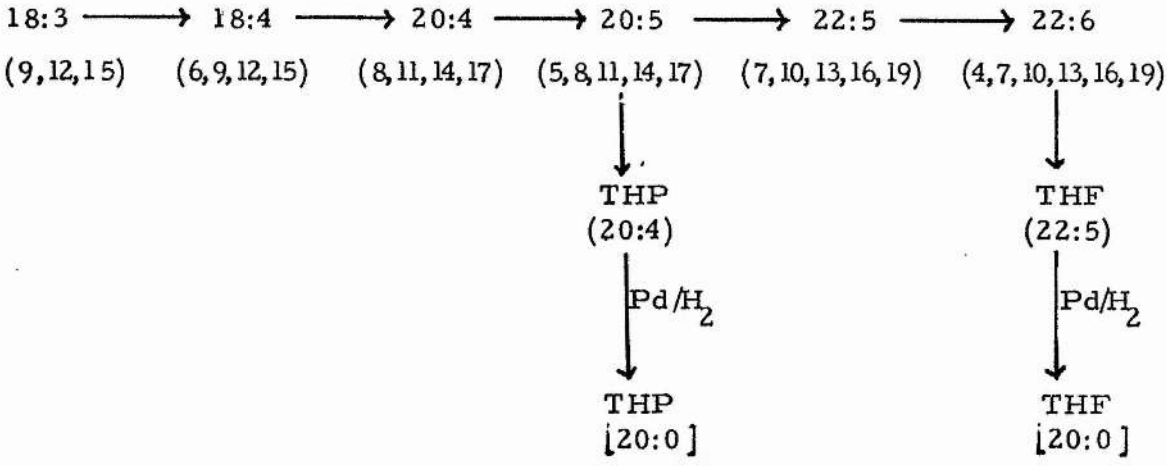
[a] 197, 198

fatty acids usually present within the n-3 and n-6 families are shown below.

n-6



n-3



Note: THP = tetrahydropyrans
THF = tetrahydrofurans

As has been pointed out previously the oxymercuration-demercuration reaction giving rise to cyclic ethers is specific to unsaturated alcohols with a Δ3t, Δ4 and Δ5 unsaturated centre. From cod liver oil, four main component alcohols should undergo the oxymercuration-demercuration process forming tetrahydropyran or tetrahydrofuran

ethers as shown above.

(a) Tetrahydropyrans

The monoalkylated tetrahydropyrans are known to be less polar than the analogous tetrahydrofurans and are therefore likely to be in band 1. This contains a dominant component (92%) of ECL 20.0 which concentrates in the second band on silver ion chromatography and has an ECL of 16.8 after complete hydrogenation. By comparison with data obtained from arachidonyl alcohol (Table 18) it seems likely that this ether has been produced from the 20:5 (5,8,11,14,17) alcohol.

The tetrahydropyran band also containns some minor components. The product of ECL 18.8 (3.4%) concentrates in the first band on silver ion chromatography (37.4%) and is therefore less unsaturated than the major 20:5 compound. Unfortunately it is not very clear what happens to this on hydrogenation. It could be the product from arachidonic acid.

The minor component of ECL 17.8 (2.6%), chromatographs with the 20:5 product on silver ion tlc (2.3%) and is hydrogenated to a product of ECL 15.0 (1.9%). This glc and tlc data suggest a C_{18} pentaene with $\Delta 5$ unsaturation. The $\Delta 5,8,11,14,17$ isomer is possible but not proven.

(b) Tetrahydrofurans

Tetrahydrofurans are concentrated in the second band from the silica tlc. This consists almost entirely (97.5%) of an ether (ECL 23.6) which concentrates in the second band on silver ion chromatography and has an ECL of 19.8 on hydrogenation. This is consistent with the products resulting from 22:6 (4,7,10,13,16,19). It was not possible to identify the minor products also present.

The isolation and detection of these acids from cod liver oil by oxymercuration-demercuration procedure was purely analytical, and quantitative analyses of these acids was not undertaken. However, the 20:4 ether was the predominantly tetrahydropyran while the 22:5 tetrahydrofuran ether comprised almost the whole of the tetrahydrofuran fraction. This result therefore confirms the predominance of n-3 over n-6 series of polyene acids in cod liver oil.

2.7.4 Mass spectrometry

The cyclic ethers isolated from cod liver oil and the cyclic ether (20:3 THP) derived from arachidonyl alcohol were subjected to mass spectrometry. The molecular ions of these cyclic ethers could reliably be identified as shown:

Molecular ion (MH^+) (hydrogenated)	Molecuar ion (M^+)	Cyclic ethers	Possibly derived from
296 (6%)	290 (6%)	20:3	20:4 n-6 (authentic)
296*	288 (1.3%)	20:4	20:5 n-3
324 (1%)	314 (2%)	22:5	22:6 n-6

* Molecular ion not displayed

The fragmentation patterns displayed by the cyclic ethers isolated from cod liver oil and that of the authentic cyclic ether (20:3 THP) could not be related to their structures.

2.7.5 Summary

The glc and tlc behaviour of these compounds and the mass spectroscopic evidence of the molecular ions led to the following

conclusions.

(a) These compounds are likely to be cyclic ethers.

(b) The 20:4 cyclic ether is probably a tetrahydropyran while the 22:5 cyclic ether is probably a tetrahydrofuran.

(c) The presence of a cyclic ether (ECL 18.8, SP2340) identical with the authentic 20:3 cyclic ether derived from arachidonic acid indicates that this may also be present in cod liver oil.

The cyclic ethers 20:3 THP, 20:4 THP and 22:5 THF must have been derived from 20:4 n-6, 20:5 n-3 and 22:6 n-3 alcohols respectively.

2.8 CONCLUSION

A detailed examination and structural identification of the various fatty acids, especially the minor components, of the cod liver oil has hitherto not been undertaken. In this study, several acids have been isolated and structurally identified by a combination of techniques, including low temperature crystallisation, urea fractionation, chromatography, nmr, uv, and mass spectrometry.

The straight-chain saturated fatty acids in cod liver oil account for about 16% of the total fatty acids of which palmitic acid is the major component (10%). Myristic acid (3.4%) and stearic acid (2.5%) constitute the second and third largest components respectively. Other straight-chain saturated acids (C_{20}, C_{22} and C_{24}) and the odd-numbered straight-chain acids (C_{15}, C_{17} and C_{19}) are present in trace amounts. The structure of the major straight-chain fatty acids (C_{14}, C_{16} and C_{18}) was confirmed by mass spectrometry.

The phytol-based acids (isoprenoids) of cod liver oil constitute about 0.5 to 1% of the total fatty acids. Of these, phytanic acid (P_{20}) makes up about 70% while pristanic acid (P_{19}) and 4, 8, 12-trimethyltridecanoic acid (P_{16}) are present in almost equal amounts. The P_{17} (5, 9, 13-trimethyltetradecanoic acid) though tentatively identified, is present only in trace amount. The structures of these phytol-based acids were confirmed by both mass spectrometry and nmr spectroscopy as follows:

P_{16} methyl 4, 8, 12-trimethyltridecanoic acid

P_{17} methyl 5, 9, 13-trimethyltetradecanoic acid

P_{19} methyl 2, 6, 10, 14-tetramethylpentadecanoic acid

P_{20} methyl 3, 7, 11, 15-tetramethylhexadecanoic acid

The furanoid acids in this oil occur at about 0.1 to 1% of the total fatty acids. Of these, F6 constitutes over 50% while F4 and F2 are significant. The various furanoid acids (F1 to F9) previously reported to occur in other fish lipids were identified in this project. Structures F1-F6 are proved by mass spectrometry, while F7-F9 are tentatively identified by the glc. The ECL of these acids are similar to those published in the literature. While this research was in progress, the structures of F5 and F6 were confirmed by synthesis, thereby confirming the structures of these acids. In addition to these acids (F1-F9) other hitherto unknown furanoid acids from the cod liver oil were isolated and characterised by GC-MS. These furanoid esters and those already identified from various sources are detailed in section 2.3, (p. 50).

The monoene acids make up about 50-55% of the total fatty acids of which the 18:1 constitutes about 22%. Also present are 20:1 (13.8%), 22:1 (8.6%), 16:1 (1.5%), and 14:1 (< 1%). Some odd-numbered monoene acids present in trace amounts are also tentatively identified.

The presence of three n-7, three n-9 and two n-11 monoenes are observed in the cod liver oil. Of these, the n-9 monoene acids are present in largest amounts (28%), followed by the n-11 (8.5%) and the n-7 (7%). The $\Delta 9$ isomers are the main components among the C_{16} and C_{18} monones and the $\Delta 11$ isomers are the main components among the C_{20} and C_{22} monoenes.

A group of mono- and diunsaturated branched-chain fatty acids have been isolated and characterised by GC-MS. A GC-MS analysis of the perhydro-derivatives of these acids revealed the presence of

the following acids: 5-methyltetradecanoic, 5,7-dimethyltridecanoic 7-methylhexadecanoic, 5,7-dimethyltetradecanoic, 7,9-dimethylhexadecanoic, 7,9- and 9,11-dimethyloctadecanoic acids. Of these, only the 7,9-dimethylhexadecanoic acid was fully characterised in its unhydrogenated form as 7,9-dimethylhexadecadienoate (6.8) . Such a conjugated branched-chain fatty acid is novel. The presence of the branched methyl groups on alternate odd carbon atoms is uncommon, but it is noted that 7-methylhexadec-7-enoic acid has already been isolated from some marine lipids.

The diene fatty acids in cod liver oil are very small in amount ($<1\%$). However, in the diene band 18:2 is the major component (70%) followed by 16:2 (17%) and 20:2 (11%) . The C_{14} and C_{22} diene acids are observed in trace amounts. The $\Delta 9,12$ isomer is the main components among the C_{16} and C_{18} dienes, while in the C_{22} diene acid the $\Delta 10,13$ isomer predominates.

The identification and characterisation of the more highly unsaturated acids revealed the presence of 20:4 n-6, 20:5 n-3 and the 22:6 n-3. The isolation and detection of these acids was purely analytical. Among these polyene acids, the n-3 series predominate over the n-6 series, a result which is consistent with published data.

EXPERIMENTAL

3.1 GENERAL PHYSICAL AND CHEMICAL PROCEDURES

(i) Purification of solvents

All solvents were of reagent grade and redistilled before use. Diethyl ether was dried by standing over calcium chloride. After decantation and distillation, it was stored over sodium wire. Pyridine was dried by refluxing with potassium hydroxide pellets. Dimethylformamide (DMF) was dried by the removal of water as an azeotrope (b.p. 78°) with benzene. The DMF was subsequently distilled (b.p. 153°) and stored over molecular sieve (type 4Å). Maleic anhydride was heated with benzene (2 g/10 ml) on the mantle to about 80-85° in the fume cupboard and quickly filtered while hot. The filtrate was evaporated in vacuo and the product crystallised on standing at room temperature. This was carefully dried and stored in an air-tight container.

(ii) Spectroscopy

Uv spectra were recorded on a Unicam SP800B spectrophotometer. Samples were run in cyclohexane solution using silica quartz cells of 1 cm path length.

Mass spectra were recorded with direct-probe insertion of samples into the source of an AEI MS902 mass spectrometer. The source pressure was 2×10^{-7} torr, source temperature about 200°C, and the ionisation voltages 70 eV.

PMR spectra were recorded at 360 MHz on a Bruker spectrometer (University of Edinburgh) using 10-15% solutions in carbon tetrachloride with 3% tetramethylsilane as the internal standard. Chemical shift values are given in ppm downfield from tetramethylsilane ($\delta = 0$).

Natural abundance ^{13}C nmr spectrum was obtained with a WH360 Bruker spectrometer (University of Edinburgh). The sample was run in deuterochloroform solution (~ 2 ml) with tetramethylsilane (3%, v/v) added as an internal standard. The spectrometer's computer printed out peak heights and chemical shifts (ppm downfield from tetramethylsilane).

(iii) Chromatographic Techniques

a) Thin Layer Chromatography (tlc)

Analytical tlc was carried out on glass plates (20 cm x 5 cm) coated with a layer of silica gel G (0.25 mm or 0.5 mm wet thickness) or with silica gel G containing 10% silver nitrate (Ag^+tlc). For monitoring column chromatography, microscope slides, coated by dipping in a slurry of silica gel G in chloroform (0.25 g/ml) and dried in air, were used. Preparative tlc was carried out on glass plates (20 cm x 20 cm) coated with silica gel G (0.5 mm or 1 mm thickness). After their preparation, the tlc plates were dried at room temperature for 15 minutes, activated at 110-120° for 2 hr and stored in a drying cabinet containing silica. Preparative silver nitrate tlc plates (0.5 mm and 1.0 mm thickness, 10% and 20% silver nitrate) were prepared, activated at 110-120° for 1 hr and stored in the drying cabinet away from light. Where necessary preparative tlc plates (tlc silica + Ag^+tlc) were pre-cleaned with distilled ether.

Mixtures of petroleum ether (b.p. 40-60°) and diethyl ether were used as developing solvents for tlc except where otherwise indicated. Abbreviations such as PE10 indicate mixtures of petroleum and diethyl ether in a volume ratio of 90:10.

Components separated on analytical plates were made visible by spraying with an ethanolic solution of phosphomolybdic acid (10%, v/v), followed by heating at 110-120° for 10 minutes. Preparative tlc plates were sprayed with an ethanolic solution of 2',7'-dichlorofluorescein (0.2%, v/v) and viewed under uv light. The resolved bands were scraped off the plate, and extracted with diethyl ether or with chloroform-methanol (2:1, v/v) for polyene esters. The solvent was removed under reduced pressure, and traces of water removed by azeotropic distillation with benzene. The residual material was finally dried under nitrogen.

b) Gas-Liquid Chromatography (glc)

Glc was carried out on a Pye series 104 chromatograph equipped with a flame ionisation detector. Glass columns (1.5 m long, 6 mm external diameter) containing 5% ApL, 10% SP2340, 20% DEGS, 3% OV210, 10% OV225 and OV17 (14:1, ratio w/w) or 20% EGSS-Y coated on Chromosorb W AW (100-120 mesh) supports were used. Isothermal analysis was carried out at various temperatures between 174° to 220°C and temperature programmed analysis in the range 40-220°C. Samples were injected directly on to the column using a 5μl or 10μl SGE syringe with a 5 cm or 11 cm needle respectively. The carrier gas (oxygen free nitrogen) flow rate was varied from 10 to 22 psi (25-75 ml/min).

Peak areas were measured by one or more of the following methods: (a) peak height x peak width at half height, (b) peak height x retention time, and (c) Du Pont curve analyser. The composition of mixtures is reported as weight percentages or as mole percentages in appropriate cases.

Saturated straight-chain methyl esters were used as external standards and ECL values and percentage areas were calculated graphically and/or by computer. The ECL values reported here are fairly consistent and appropriate authentic samples were chromatographed periodically to check the variation of ECL values, due either to column ageing or temperature fluctuation.

c) Preparative glc

Preparative glc was carried out on a Pye series 104 chromatograph equipped with a flame ionisation detector and a collecting device. The glass column (1.5 m long x 5 mm external diameter) contained either 3.5% ApL or 20% EGSS-Y, coated on chromosorb W, AW (100-120 mesh) support. The oven temperature was 200° and nitrogen (22 psi) was used as carrier gas while hydrogen (15 psi) and air (10 psi) were used for the flame ionisation detector.

The outlet temperature (independently controlled) was varied between 195 and 210° according to the nature of the substance under investigation, and a split ratio of 100:1 in favour of the collector was used. Separated components were collected in chloroform (0-10°) using glass tubes (90 mm length) fitted at the bottom end with sintered glass cylinders (12 mm x 25 mm) of coarse porosity. The volume of chloroform in each collecting vial was adjusted and maintained to give a split ratio of 100:1 in favour of the collector.

Samples (10μl containing 0.5-0.7 mg/μ l) were injected. Four separate injections were sufficient to collect components adequate for further analyses. Distilled hexane was injected between each peak to improve purity. Chloroform in each vial was evaporated to dryness by a gentle stream of nitrogen. Esters in

each fraction were weighed and checked for purity on analytical columns (both ApL and SP2340)

(iv) Other separation procedures

a) Urea fractionation

Free fatty acids or methyl esters were crystallised at 0°C overnight from a solution of methanol containing urea. After filtration and washing with methanol saturated with urea, the filtrate was concentrated by heating under reduced pressure, diluted with water, acidified with 10% sulphuric acid and brine added. The acid was thoroughly extracted with diethyl ether (x 2) and the combined ether extracts were washed with water before final removal of solvent. The adduct released its acids or esters when mixed with water and these were extracted with diethyl ether (x 2) as described for the filtrate.

b) Low temperature crystallisation

Cod liver acids (100 g) were dissolved in acetone (1 l) and cooled to -20°C in a Dewar containing acetone and cardice. After 5 hr the solution was filtered through a Buchner funnel cooled by solid cardice to the crystallising temperature (-20°C). The precipitate was carefully pressed with a glass stopper and washed with pre-cooled acetone (-20°C). The acids were subsequently recovered from the precipitate (ppt A, 6.4 g). The mother liquor was subjected to the same crystallisation procedure at -40°C for 5 hr to obtain precipitate B (4.3 g). The mother liquor was further subjected to crystallisation at -55°C to obtain precipitate C (17.7 g). The mother liquor was finally subjected to crystallisation at -75°C to obtain precipitate D (25.6 g) and mother liquor D (28.0 g).

(v) General chemical procedures

a) Preparation of mixed acids (FFA)

Cod liver oil (105 g) was refluxed for 1 hr with a solution of potassium hydroxide (34 g in 40 ml water and 200 ml ethanol). After cooling, the mixture was poured into a separating funnel containing crushed ice (150 ml) followed by sulphuric acid (20% conc, 210 ml). The fatty acids were extracted with petroleum ether (b.p. 40-60°, 2 x 150 ml) and the petroleum extracts were washed with distilled water (3 x 100 ml) and then filtered before removal of the solvent. The residue was dried by azeotropic distillation with benzene (40 ml) followed by a stream of nitrogen to obtain the free acids (100 g).

An aliquot of the saponified cod liver oil (FFA) and unsaponified oil as control were each applied to the tlc plate and developed in PE20 for 5 mins. The plate was dried and sprayed with 10% phosphomolybdic acid in ethanol and incubated at 110° for 5 min. A single discrete spot showed the location of free fatty acids and absence of other products.

b) Preparation of methyl esters (small scale)

Acids (50 mg) were refluxed with acidic methanol (2 ml), containing 2% concentrated sulphuric acid) for 30 min. After cooling, brine (5 ml) and then petrol ether (b.p. 40-60°) were added and the mixture shaken. The petrol layer was carefully pipetted off and retained. The extraction was repeated with more petrol (5 ml). The combined extracts were dried over anhydrous sodium sulphate and the solution finally filtered or decanted. About 3μl amount was injected directly into the glc for the total analysis of the esters.

c) Transesterification (base-catalysed)

Lipid (1 g) was dissolved in benzene (3 ml) and refluxed with freshly prepared sodium methoxide in methanol (0.5M, 6 ml) for 2 hr. The esters were recovered in the usual manner as described above for the acid-catalysed esterification. This procedure was also carried out on a larger scale.

d) Hydrogenation

The unsaturated ester (5-10 mg) was dissolved in methanol (2-3 ml) in a round-bottomed flask (25 ml, vol) and palladium on charcoal (10%, 1-2 mg) and a small magnetic paddle were added. The flask, fitted with a two-way tap, was alternatively evacuated and flushed with hydrogen several times to remove air, and finally opened to the hydrogen atmosphere. The reaction mixture was vigorously stirred for 1 hr (or overnight if preferred) at room temperature. The catalyst was removed by filtration and the residue washed with warm solvent (ether or petrol) and the material recovered by evaporation under nitrogen.

e) Trimethylsilylation

Alcohols were converted into their trimethylsilyl ethers (TMS ethers) to facilitate examination by the glc. To ca. 5 mg of material in pyridine (1 ml) was added hexamethyldisilazane (HMDS, 0.2 ml) and trimethylchlorosilane (TMCS, 0.1 ml). After 5 min, the pyridine was removed under vacuum and the TMS ethers dissolved in diethyl ether.

Note: The cod liver oil used in these studies was supplied by the Marfleet Refining Company and this was supplemented by purchase of cod liver oil containing highly PUFA from a local pharmacist.

3.2 STRAIGHT-CHAIN SATURATED ACIDS

(i) Urea fractionation

Cod liver oil free fatty acids (50 g) were crystallised at 0°C from methanol (200 ml) containing urea (50 g). The acids in the adduct (A1, 28 g), treated in the same way (urea 112 g, methanol 450 ml), gave a second adduct (A2, 21.5 g).

(ii) Silver ion chromatography

This second adduct 2 was separated by silver ion tlc (100 mg/plate, 0.5 mm thickness) using PE5 as developing solvent. The first band (A2AgF1) contained saturated straight-chain acids (C_{12}-C_{20}) and the second (A2AgF2) was mainly monoenes.

3.3 ISOPRENOID ACIDS

(i) Transesterification - in sodium methoxide solution

Cod liver oil (1400 g) dissolved in benzene (1500 ml) was refluxed for 3 hr with methanolic sodium methoxide solution (0.5 %, 2 l.). After addition of acetic acid (200 ml) and brine (1.5 l.) the esters were extracted with distilled petroleum ether (b.p. 40-60°, 2 x 1 l.) and the petrol extracts were washed first with brine (1 x 2 l.) and then with distilled water (2 x 1.5 l.). Removal of solvent furnished cod liver methyl esters (1450 g).

(ii) Urea fractionation

Cod liver methyl esters (1450 g) were crystallised at 0° from methanol (3.6 l.) containing urea (2900 g). The esters (266 g) remaining in the liquor, treated in the same way (urea 1330 g,

methanol 1330 g) gave a second liquor which contained 111 g of esters (see Table 2, section 2.2).

(iii) Silver ion chromatography

A column of silica (52 x 9.5 cm) containing 10% silver nitrate wrapped in aluminium foil to exclude light, was equilibrated with PE5 prior to addition of the second mother liquor (100 g). Material was eluted with PE5 (1.5 l., 3.0 g), PE10 (1.5 l., 10.5 g) and ether (1.5 l.).

(iv) Thin layer chromatography

Preparative argentation tlc was used to separate isoprenoid acids from other branched-chain compounds. Fraction 1 (AgCCF1) already contained only isoprenoid acids. Fraction 2 (AgCCF2, 200 mg on each 7 silver ion plates) was developed in PE7.5, sprayed with ethanolic 2',7'-dichlorofluorescein reagent and viewed under the uv light. Authentic methyl phytanate, 18:1 and 18:0 were developed alongside the sample to help locate appropriate bands. Five separate bands were obtained: (1) mainly hydrocarbons, (2) mainly saturated esters, (3) mainly isoprenoid esters and a small amount of furanoid esters, (4) mainly furanoid esters with other branched-chain compounds and (5) probably branched-chain monoenes.

Isolation and purification of the isoprenoid esters was finally carried out on band 3 using silver ion tlc plates (100 mg/plate) of 0.5 mm thickness developed in PE5. Three distinct bands were observed. Band 2 contained isoprenoid esters only.

(v) Column chromatography (silica)

Sorbsil silica gel (100 g) packed into a glass column (1.5 x 120 cm) was equilibrated with distilled petroleum (b.p. 40-60°). AgCCF2 (2.5 g) dissolved in petroleum ether was applied to the column and eluted with 100 ml portions of PE1, PE2, PE5, PE10, PE20 and PE50, followed by ether. The progress of elution was monitored by tlc (silica) using phosphomolybdic acid as the detecting agent. Methyl esters were eluted with PE5 and PE10 after non ester impurities had been eluted with PE1 and PE2.

3.4 FURANOID ACIDS

(i) Transesterification - in sodium methoxide solution (see section 3.3 (i)).

(ii) Urea fractionation

Cod liver oil methyl esters (400 g) crystallised at 0° from methanol (1.8 l.) containing urea (800 g) furnished a mother liquor (102 g) and an adduct 1 (220 g).

The urea crystallisation procedure was repeated on the mother liquor in the ratio of 1:5:5 (methyl ester:MeOH:urea) and left at 0° for 48 hr. The second mother liquor (32.0 g) was recovered as already described. The two adducts released their methyl esters (220 g and 70 g respectively) when mixed with water.

(iii) Argentation chromatography

a) Silver column chromatography

The adsorbent (silica gel containing 30% silver nitrate) was packed into a chromatographic column in the conventional way and this was wrapped in aluminium foil to exclude light. The column was equilibrated with PE5 and the sample (30 g, 2nd mother liquor) applied and eluted with successive portions (100 ml) of PE5 (x 5), PE10 (x 3), hexane:benzene (1:1), PE20 and PE30. The progress of elution was followed by tlc on microscope slides with phosphomolybdic acid as charring agent. Altogether, 11 fractions were obtained. After examination by thin layer chromatography the first eight of these were combined for further studies.

Fraction	weight (g)	% by wt of ML	Solvents used (100 ml each)
F1	0.20	0.66	PE5
F2	0.40	1.33	PE5
F3	0.40	1.33	PE5
F4	0.20	0.66	PE5
F5	0.03	0.10	PE5
F6	0.04	0.13	PE10
F7	0.05	0.16	PE10
F8	0.05	0.16	PE10
F9	1.00	3.33	Hexane:Benzene (1:1)
F10	1.30	4.33	PE20
F11	0.75	2.50	PE30

b) Silver ion tlc

About 50 mg samples of combined fractions (CCF1) were applied to each Ag^+ tlc plate as a streak and dried under nitrogen. The plates were developed in PE7.5, dried in a gentle stream of nitrogen and sprayed with an ethanolic solution of 2',7'-dichlorofluorescein (0.2 %). The separated components (5 bands) were viewed under the uv light. Furanoid esters occurred in band 3 (very faint) between band 2 (phytanate) and band 4 (monoene).

(iv) Column chromatography

Sorbsil silica gel (50 g, 60 mesh) was packed into a column (1.5 x 65 cm) and equilibrated with distilled petrol (b.p. 40-60°). Fraction A' (1.2 g) was dissolved in petrol, applied to the column, and eluted with 100 ml portions of PE1, PE2, PE5, PE10, PE20, PE50 and diethyl ether (see later).

Fractions	Amount (mg)	Solvents used	% Recovery
CF1	100	PE1	
CF2	60	PE2	
CF3	30	PE5	
CF4	50	PE10	
CF5	350	PE20	82.5
CF6	50	PE20	
CF7	50	PE20	
CF8	300	Diethyl ether	

A purity check was carried out on all fractions using tlc plates (Ag^+ and ordinary) as already described. The methyl esters concentrated in CF5 and CF6 were combined.

3.5 OTHER BRANCHED-CHAIN ACIDS

(i) Isolation of U1 by silver ion chromatography

Methyl ester (Ag^+CCF2, see section 2.2, Scheme I, 3.5 g) was applied to a 30% silver nitrate column chromatography (52 x 9.5 cm) and eluted first with PE5 (100 ml) to give F1 (< 1 mg). Subsequent elution with benzene/hexane (1:1 ratio, v/v, 100 ml) furnished F2 (2.48 g). The column was finally eluted with diethyl ether (100 ml) to give F3 (200 mg). The F2 fraction, containing mainly U1, other branched-chain esters and furanoid esters, was applied to 20% silver ion plate (1.0 mm thickness), 200 mg/plate, and developed in PE5 to give four bands: (1) mainly isoprenoids, (2) furanoid esters and P_{16}, (3) U1 (43%), F2 (14%) and other unsaturated branched-chain esters, and (4) polyenes.

Band 3 was reapplied to 20% Ag^+tlc plates (0.5 mm thickness 100 mg/plate) and developed first in benzene and then in chloroform/methanol (99:1, v/v) in the same direction. Five different bands were obtained: (1) U1 (50%), other unsaturated branched-chain esters, and some furanoid ester, (2) branched-chain esters, (3) mainly furanoid ester (F6, > 85%), (4) very small and (5) discarded.

(ii) Preparation of pyrrolidides

A mixture of methyl ester (1-2 mg), freshly distilled pyrrolidine (0.3-0.4 ml) and glacial acetic acid (0.1 ml) was heated in a sealed tube at 100°C for 40-50 min and then cooled to room temperature. The amide so formed was taken up in methylene chloride (1.5-2 ml) and washed in 3M HCl (0.5 ml) and distilled water (0.5 ml). The washing was repeated and the sample dried

over anhydrous magnesium sulphate. The amide so formed was analysed by speetrometry.

(iii) Maleic anhydride adduct formation - Diels Alder reaction with conjugated dienes

Conjugated dienoate (70 mg) was refluxed for 3 hr with benzene (7 ml) and purified maleic anhydride (80 mg) and allowed to cool to room temperature. Solvent was removed in vacuo and the product was dissolved in ether and washed with water (4 x 30 ml). The extract was dried over anhydrous sodium sulphate, evaporated in vacuo and the product (71.2 mg) analysed by the glc. The product was applied to a pre-washed tlc plate (0.5 mm thickness) and developed in PE20 along with authentic samples of a maleic anhydride adduct 18:2 methyl ester. Three different bands were obtained. Band 1 (2.8 mg) occupied the same position as authentic conjugated diene adduct and then examined by mass spectrometry.

(iv) Stereomutation of ester mixture containing U1

The methyl esters (70 mg) were dissolved in petrol (2 ml) and irradiated with a 150 W tungsten lamp for about 4 hr in the presence of a trace of iodine. The petrol solution was washed with 0.1 M sodium thiosulphate and dried. Crystallisation in petrol at -20° overnight furnished crude diene. Repeated recrystallisation (x 2) from petrol at -20° furnished the trans,trans methyl esters.

(v) Preparation of polyhydroxy derivative of U1 by treatment with osmium tetroxide (OsO_4).

U1 methyl ester (1 mg) was dissolved in pyridine (1 ml) and OsO_4 (4 mg) were added dissolved in pyridine (0.2 ml). After 10 min at room temperature pyridine was removed by evaporation under reduced pressure. The residue was dissolved in methanol (1 ml) and saturated for 10 min with hydrogen sulphide. After centrifugation at 15,000 g and decantation of the supernatant the sediment was resuspended in methanol:water (1 ml, 1:1, v/v) and the solution was again saturated with hydrogen sulphide for 10 min. The combined supernatants were evaporated under reduced pressure and subsequently dried over phosphorus pentaoxide.

The polyhydroxy U1 methyl ester was converted to O-TMS derivative, the residue dissolved in dry methylene chloride and analysed by mass spectrometry.

3.6 MONOENOIC ACIDS

(i) Isolation

Monoene fatty acids were isolated from cod liver oil by low temperature crystallisation, preparative silver ion tlc and preparative glc (section 2.5, Scheme V).

(ii) Ozonolysis

Pure methyl ester (1 mg) was dissolved in distilled methylene chloride (0.5 ml) pre-cooled to -70°C and kept in acetone/cardice mixture (-70°) in a suitable thermostatted flask for 15 min. Oxygen/ozone mixture from a BOC Cryoproducts MKII Ozoniser

was bubbled through at the rate of 30 ml/minute for 1-3 minutes. The sample was brought to room temperature and excess ozone removed with nitrogen. The sides of the container were washed down with a few drops of methylene chloride at -70° (0.5 ml) and triphenylphosphine (20 mg/mg sample) added. The sides of the tube were again washed down with methylene chloride (0.5 ml) at -70°, and the tube was thoroughly shaken and kept at -70° for 5 to 10 minutes. The reaction mixture was brought to room temperature and excess solvent gently blown off with N_2. The products were dissolved in distilled hexane (0.5 ml) and both the aldehydes and aldehydo-esters analysed by the glc.

3.7 DIENOIC ACIDS

(i) Isolation

Mother liquor 'C' obtained by low temperature crystallisation (see section 2.5) was subjected to preparative silver ion tlc and preparative glc for the isolation of diene acids.

(ii) Ozonolysis

The diene methyl ester (1 mg) was dissolved in distilled ethanol (0.5 ml) in a Quickfit tube and immersed in a cardice-acetone mixture at -70° in a thermostatted flask. After about 10-15 min, when the methyl ester-ethanol mixture would have attained the required temperature (-70°), ozone (generated from a BOC Cryoproducts MK II at a flow rate of 50-100 ml/min) and oxygen mixture was bubbled into the diene solution for about 5 min. The appearance of a distinct blue colour indicated the end point.

The tube was quickly brought to room temperature and the solvent gently evaporated with N_2. Freshly distilled ethanol (0.2 ml) was added into the tube and the ozonolysis products were quantitatively transferred into a 2 ml reaction tube (centrifuge tube) and kept at 0° for about 5 min . Distilled water (0.1 ml) was added followed by bromothymol blue indicator solution (0.1 ml)*. Sodium borohydride (50 mg/0.1 ml) was added until the solution turned from brown to blue, followed by the addition of commercial buffer of pH 7.4 (saturated, 0.05 ml). The reaction tube was brought to 50° for 10 min after cessation of effervescence. About 3-5 μl of the sample was then injected into the glc.

(iii) Glc analysis of ozonolysis products

10% OV225 (cyanopropylmethylphenylsilicone) and 10% OV17 (phenylmethylsilicone) were separately prepared on Chromosorb W-HP (80-100 mesh) in acetone. The two phases were mixed in the ratio of 93.3% of OV225 to 6.7% of OV17 (14:1) and then packed in a glass column (1.5 m x 4 mm, i.d.) and reconditioned overnight at 300°. The efficiency of the column was 1300 theoretical plates/foot.

A temperature-programmed elution of the alcohols and alcohol esters was carried out on this OV17/OV225 column. The column was operated at 40° and raised to 240° at a rate of 4°/min. The injection port temperature was maintained at about $280\text{-}300^\circ$ with the aid of an electronic thermometer. Carrier gas (N_2) and H_2

* Bromothymol blue (0.1 g) was dissolved in 0.05M NaOH (3.2 ml) and diluted to 250 ml with distilled water.

were each set at 13 psi, and air at 15 psi. After several runs, the column was activated by the injection of HMDS (1-2μl).

3.8 OTHER POLYENES

(i) Lithium aluminium hydride (LAH) reduction

Acids/esters were converted to long-chain alcohols by reaction with excess lithium aluminium hydride (LAH) in dry ether. To a stirred suspension of LAH (200 mg) in dry ether (15 ml) was added dropwise, a solution of methyl ester/fatty acid (900 mg) in dry ether (15 ml). After stirring for 30 min at room temperature, excess hydride was destroyed by the cautious addition of wet ether (25 ml) and then water (25 ml). Dilute sulphuric acid (2M, 80 ml) was added and the product was extracted in ether (2 x 60 ml) and dried over anhydrous sodium sulphate. The recovered products (mixed alcohols) were purified by tlc (0.5 mm thickness developed in PE20) alongside authentic alcohols. The alcohols (850 mg) were recovered from the silica gel G in the usual manner.

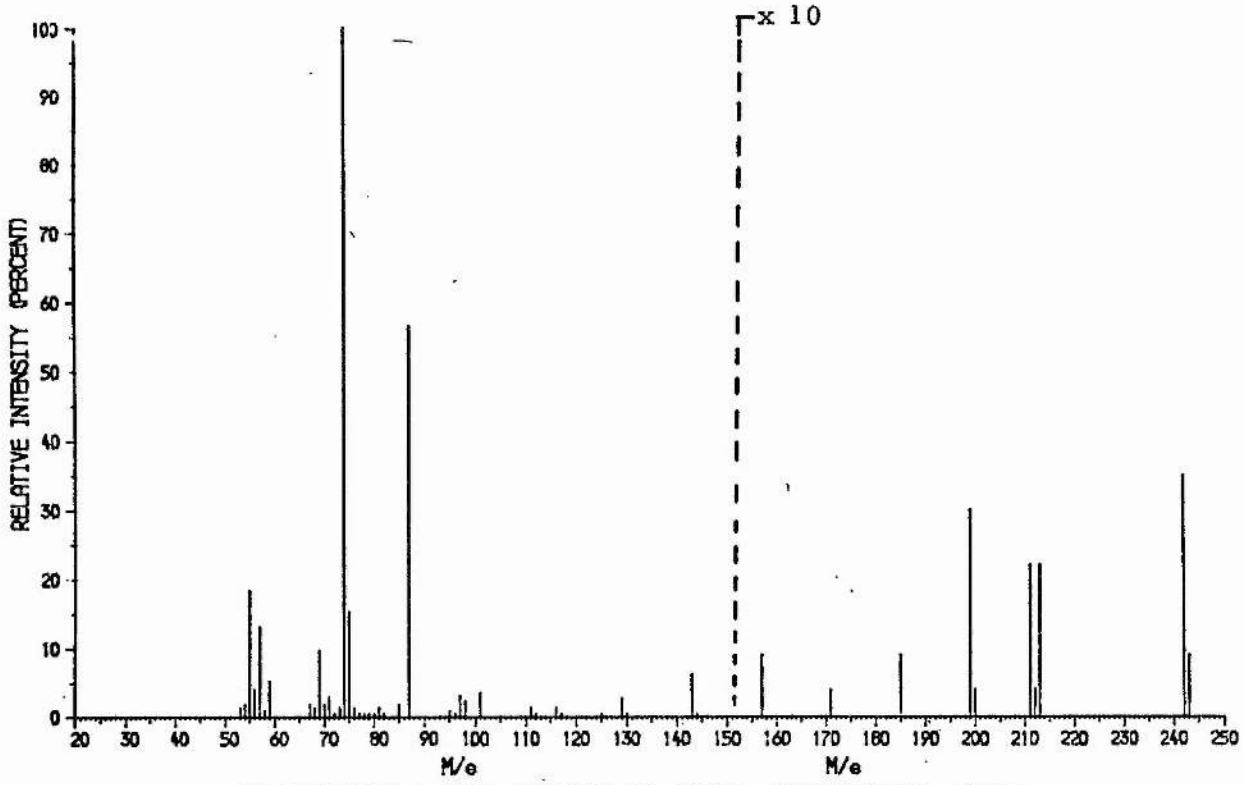
(ii) Oxymercuration-demercuration

The mixed alcohols (450 mg), mercuric acetate (900 mg-1.0 g) and DMF (10 ml) were thoroughly mixed and left at room temperature for four days. The mixture was then cooled to 0^{o} during the dropwise addition of excess sodium borohydride (300 mg) in water (10 ml). After stirring the mixture for 30 min at room temperature, the solution was saturated with sodium chloride followed by addition of water (100 ml), and the products (400 mg)

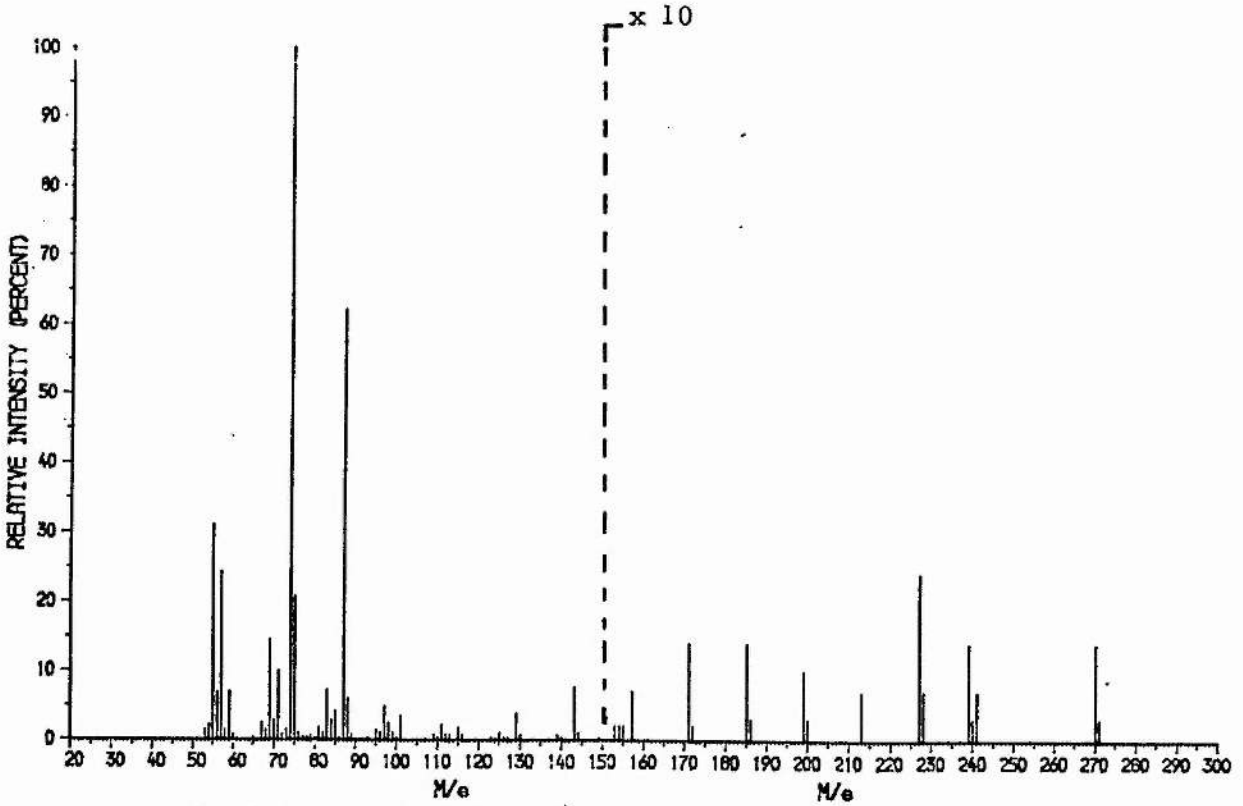
extracted with ether (2 x 50 ml). Cyclic ethers were separated from unreacted alcohols by preparative tlc (0.5 mm thickness, 100 mg/plate) developed in PE10. The cyclic ethers (the fastest bands 1 and 2) and the unreacted alcohols (near point of application) were recovered from the silica in the usual manner. The cyclic ethers were separated by silver ion tlc, bands extracted with chloroform-methanol (40 ml, 2:1, v/v) and filtered through a small Buchner funnel under reduced pressure. The silver ion-silica gels were further extracted with chloroform (2 x 20 ml).

The combined residues were dissolved in ether (30 ml), washed with distilled water (20 ml) and dilute aqueous ammonia (0.5M, 15 ml) to remove any silver nitrate and 2',7'-dichlorofluorescein. The extraction of the cyclic ethers was repeated and the extracts dried under anhydrous sodium sulphate and concentrated on the rotary evaporator. The various cyclic ethers (ca 40 mg) were finally dried under nitrogen.

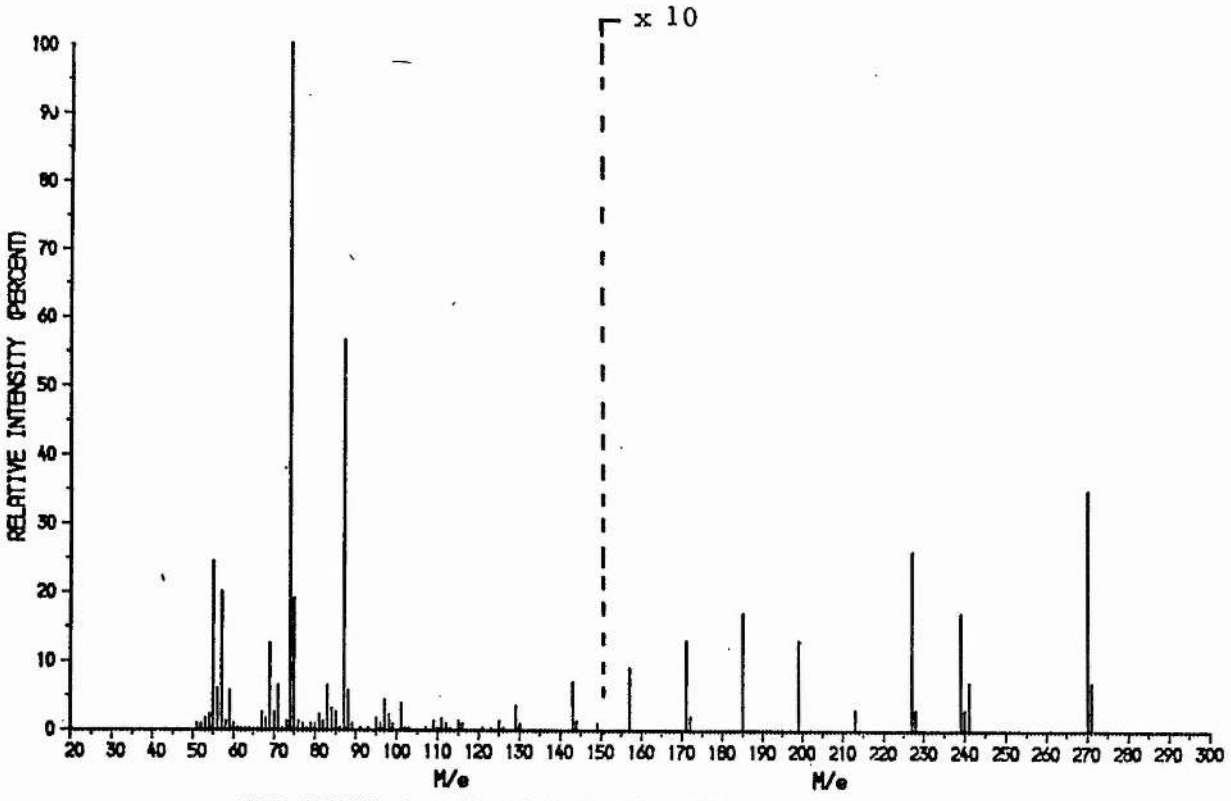
APPENDIX



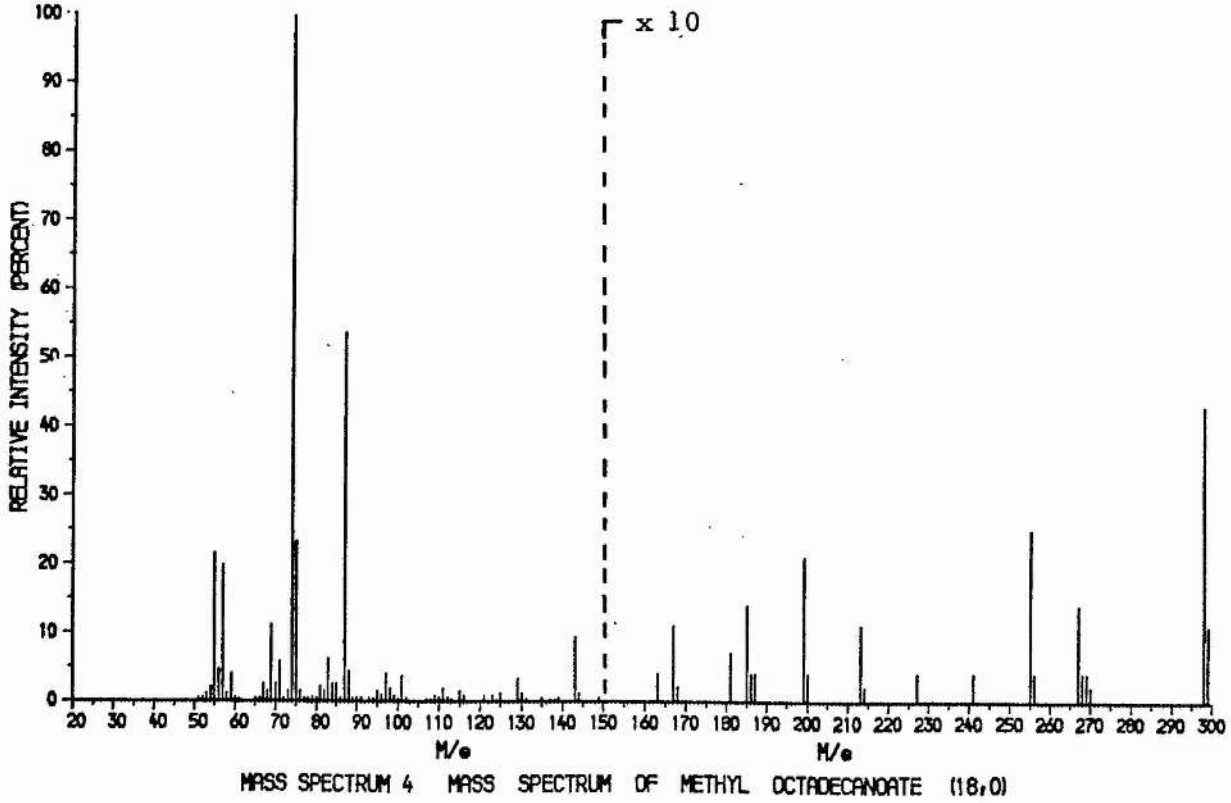
MASS SPECTRUM 1 MASS SPECTRUM OF METHYL TETRADECANOATE (14:0)



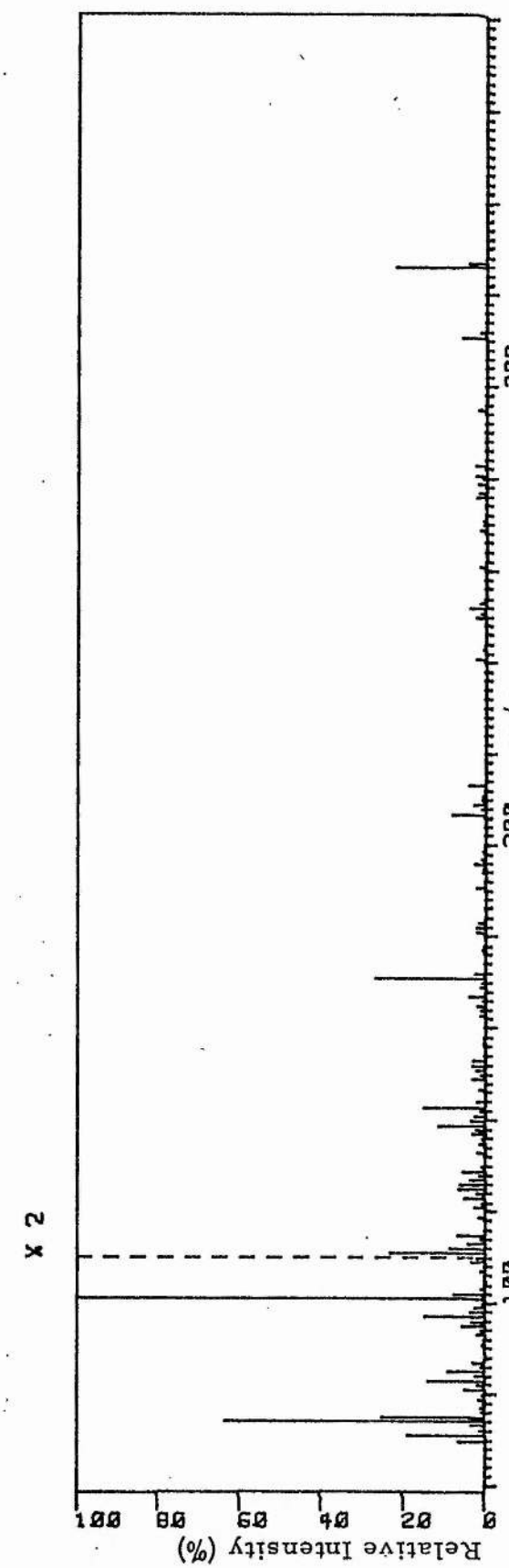
MASS SPECTRUM 2 MASS SPECTRUM OF STANDARD METHYL HEXADECANOATE (16:0)



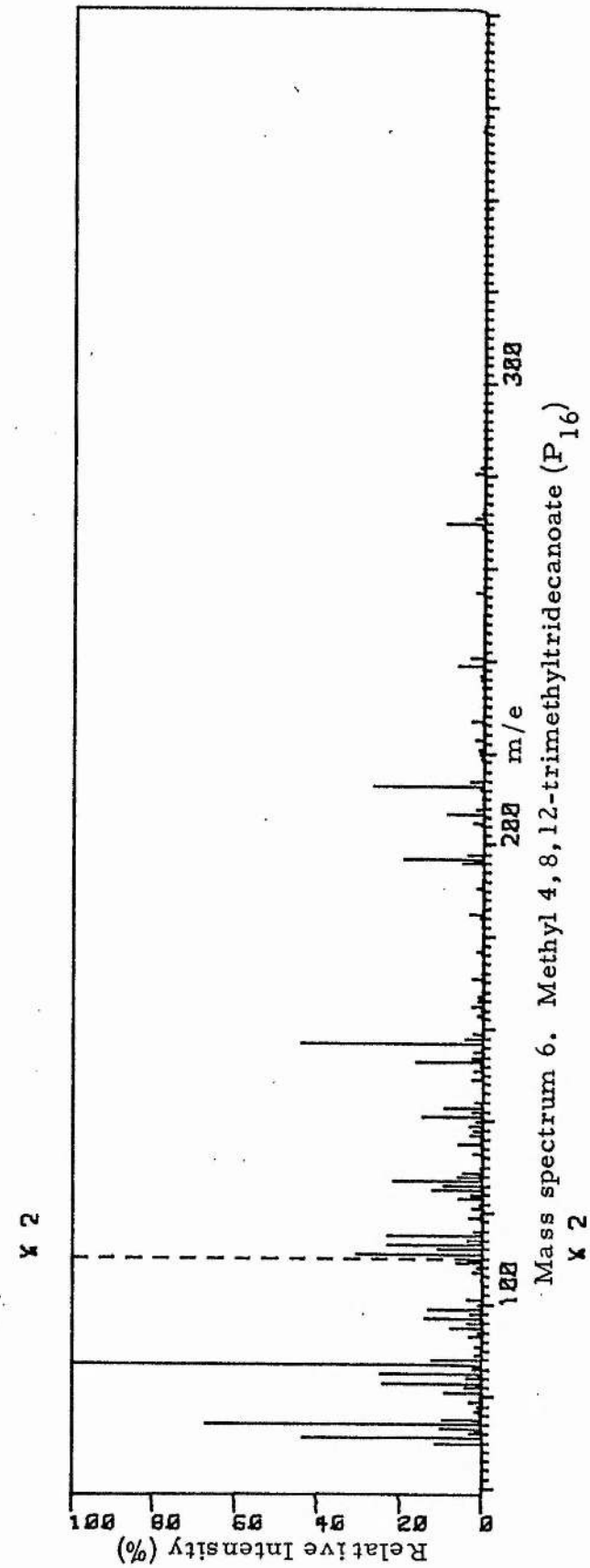
MASS SPECTRUM 3 MASS SPECTRUM OF METHYL HEXADECANOATE (16,0)



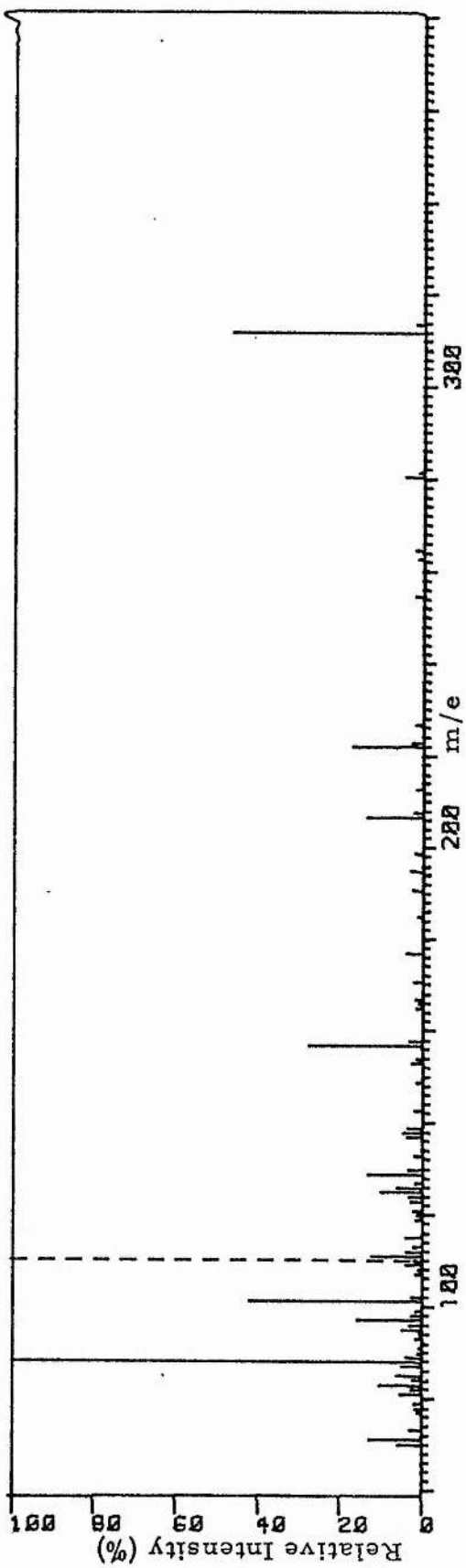
MASS SPECTRUM 4 MASS SPECTRUM OF METHYL OCTADECANOATE (18,0)



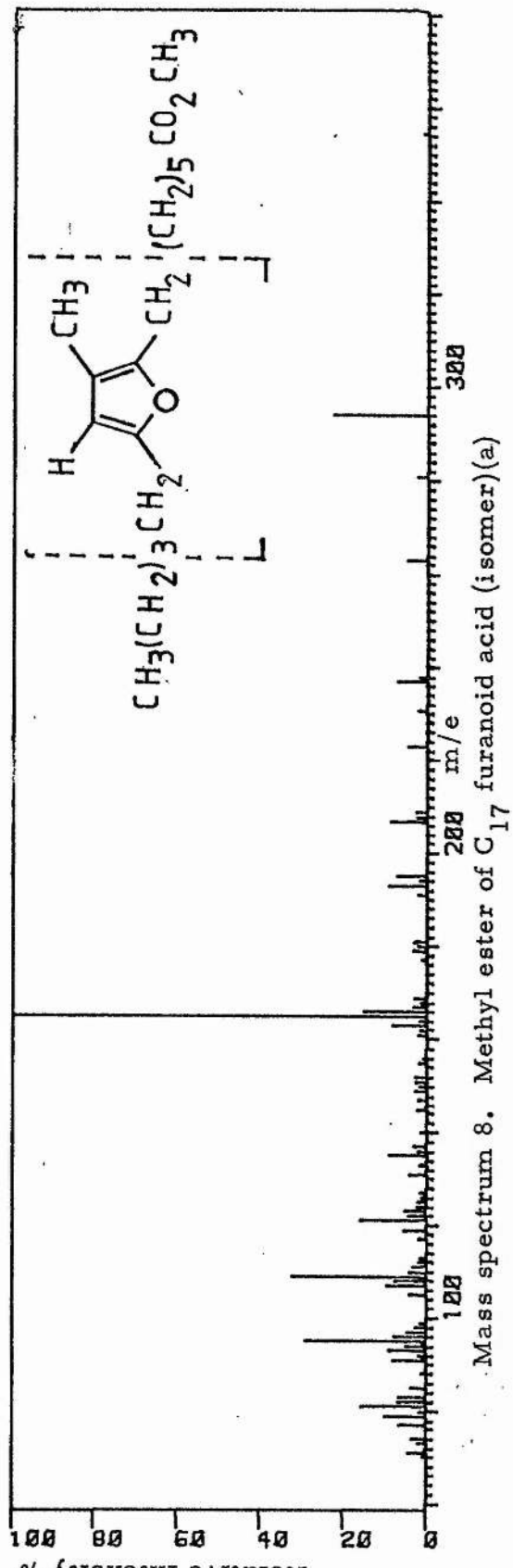
Mass spectrum 5. Methyl 3,7,11,15-tetramethylhexadecanoate (P_{20})



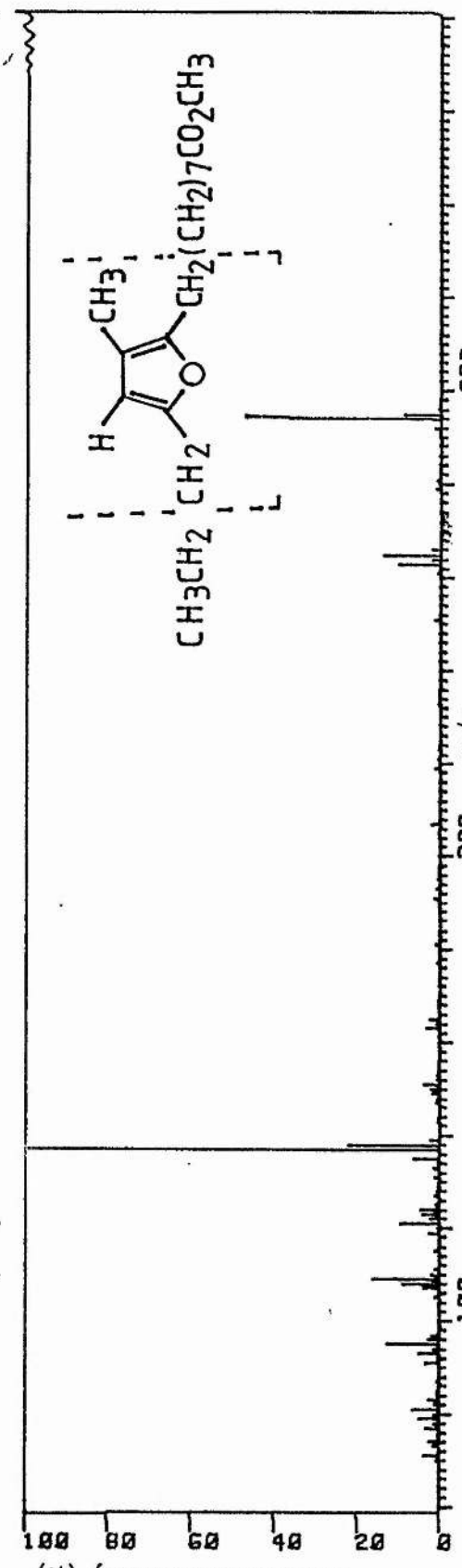
Mass spectrum 6. Methyl 4,8,12-trimethyltridecanoate (P_{16})



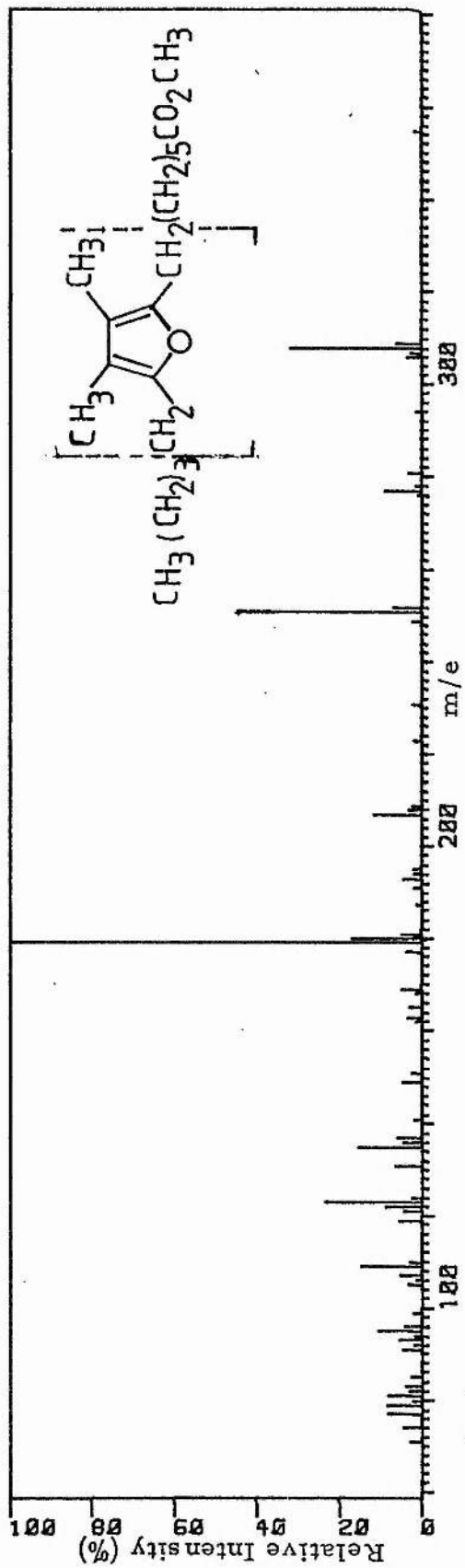
Mass spectrum 7. Methyl 2,6,10,14-tetramethylpentadecanoate (P_{19})



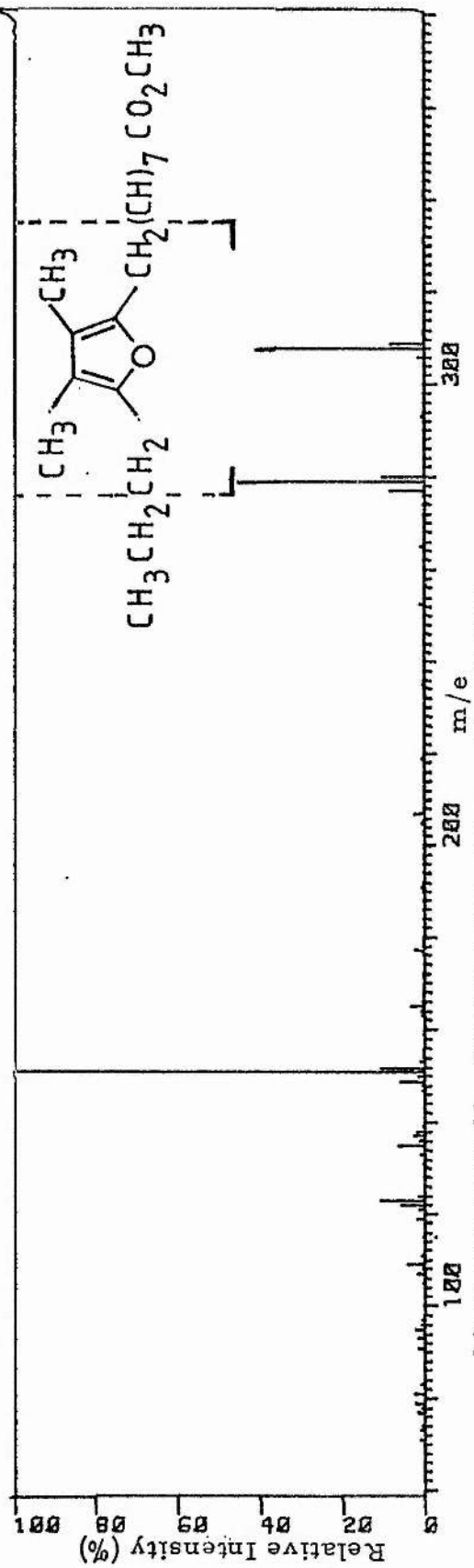
Mass spectrum 8. Methyl ester of C_{17} furanoid acid (isomer)(a)



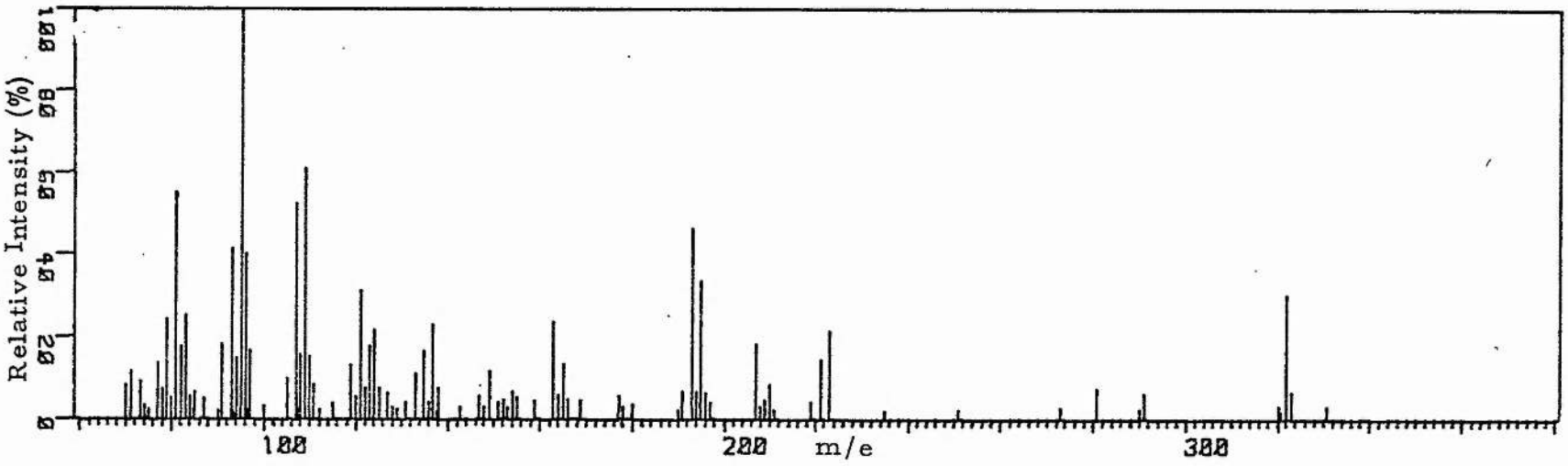
Mass spectrum 9. Methyl ester of C_{17} furanoid acid (isomer) (b)



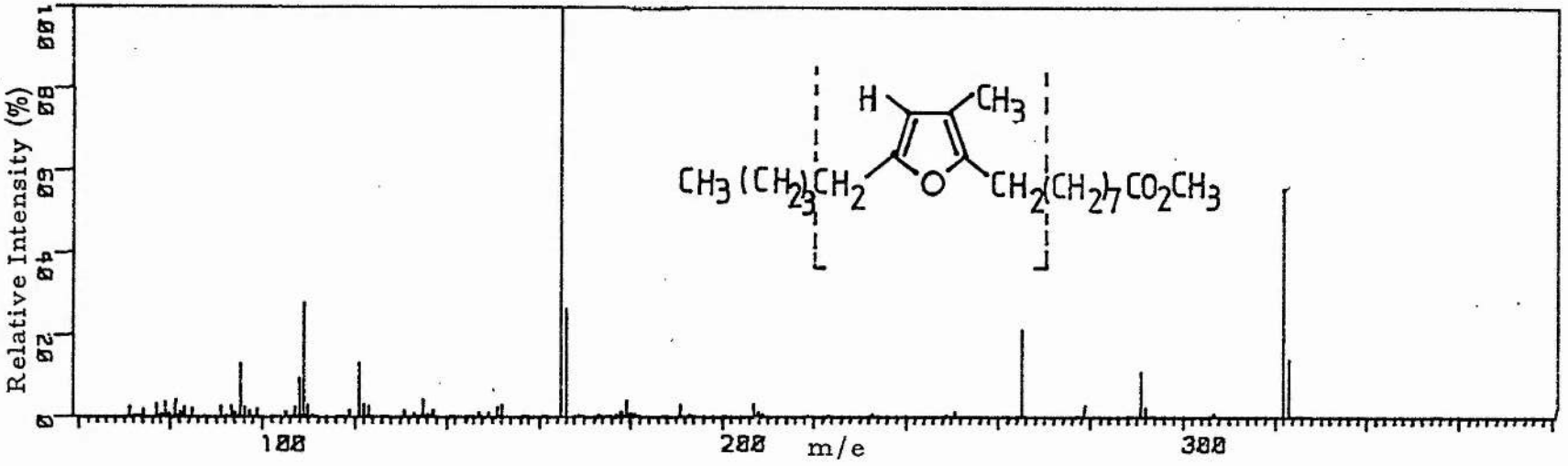
Mass spectrum 10. Methyl ester of C_{18} furanoid acid (isomer)



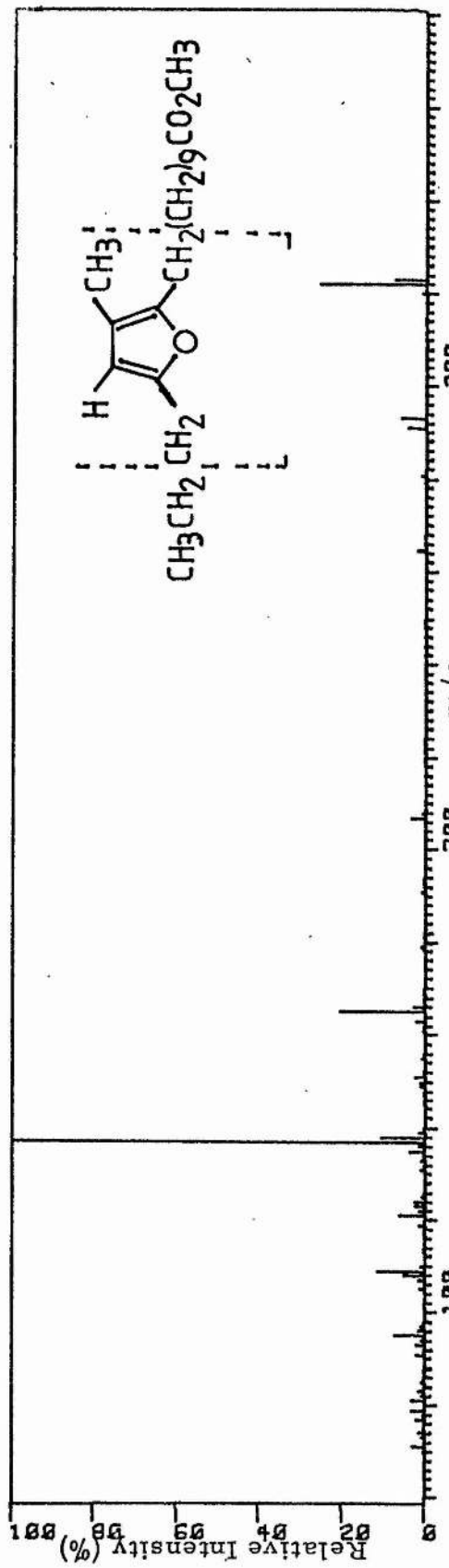
Mass spectrum 11. Methyl ester of furanoid acid (F1)



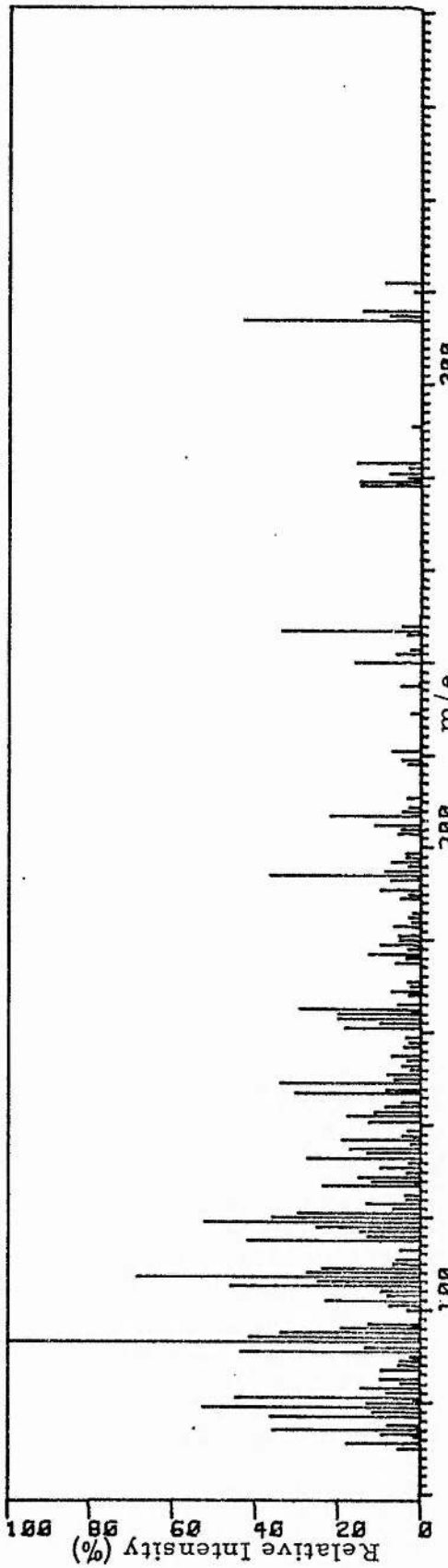
Mass spectrum 12. Methyl ester of unidentified C_{19} acid ($C_{20}H_{34}O_3$)



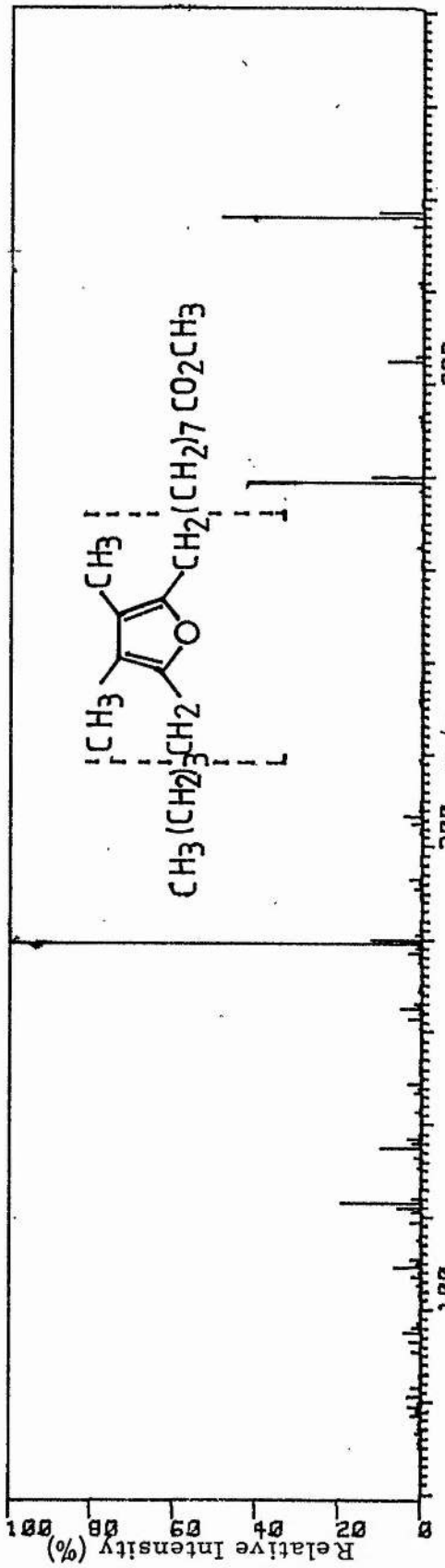
Mass spectrum 13. Methyl ester of C_{19} furanoid acid (F2)



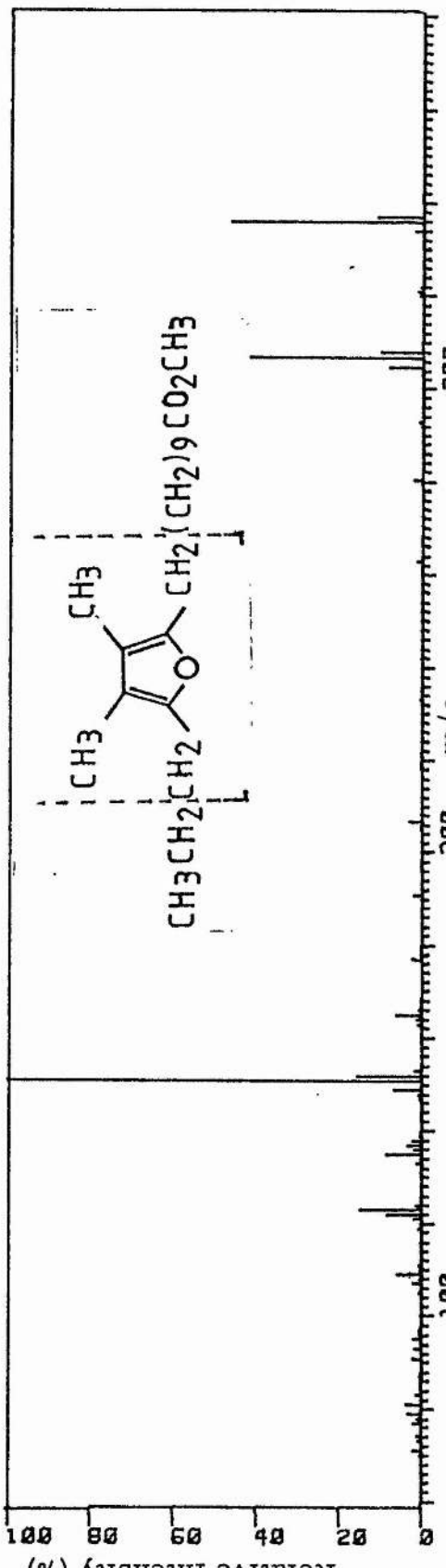
Mass spectrum 14. Methyl ester of C_{19} furanoid acid (isomer)



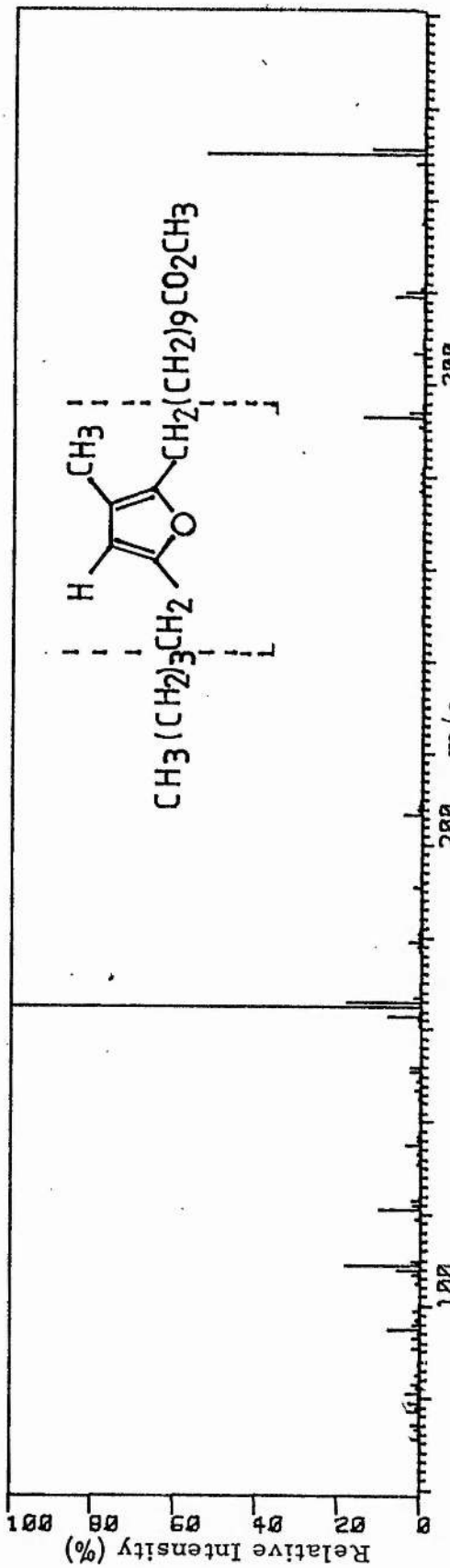
Mass spectrum 15. Methyl ester of unidentified C_{19} acid ($C_{20}H_{34}O_3$)



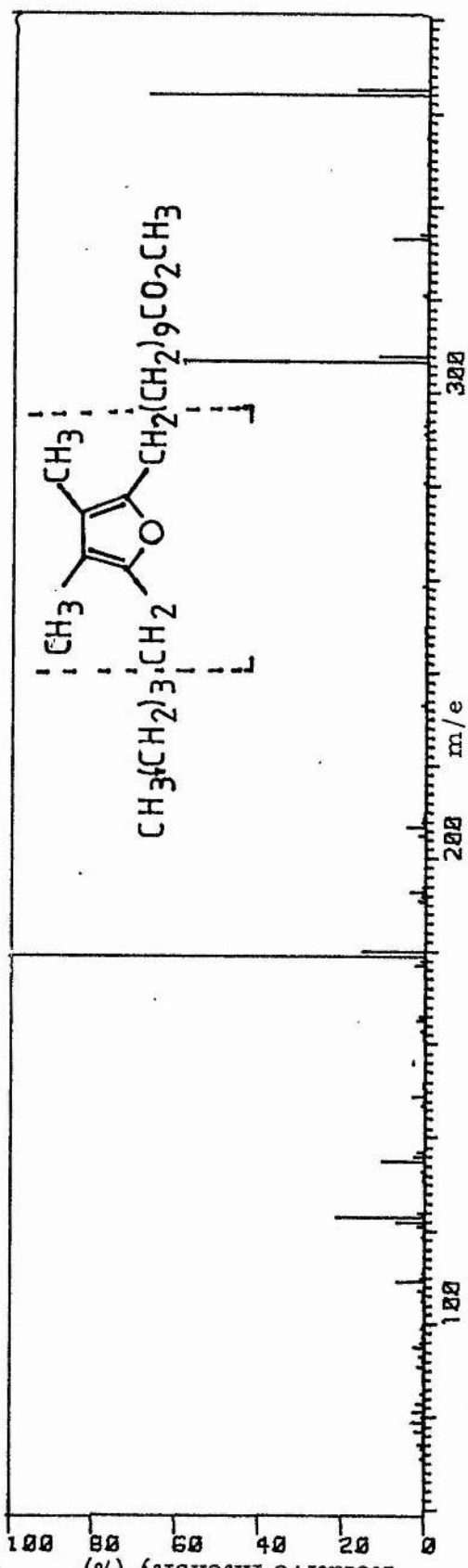
Mass spectrum 16. Methyl ester of C_{20} furanoid acid (F3)



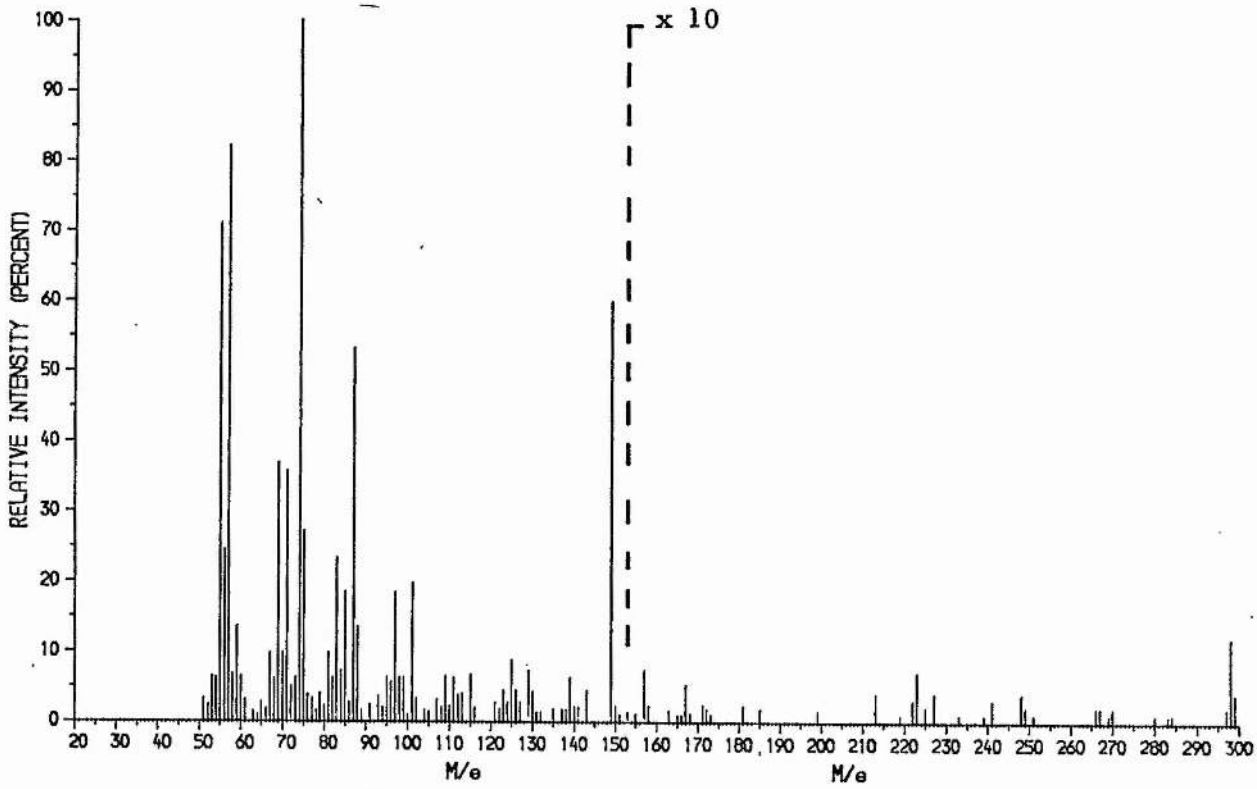
Mass spectrum 17. Methyl ester of C_{20} furanoid ester (F4)



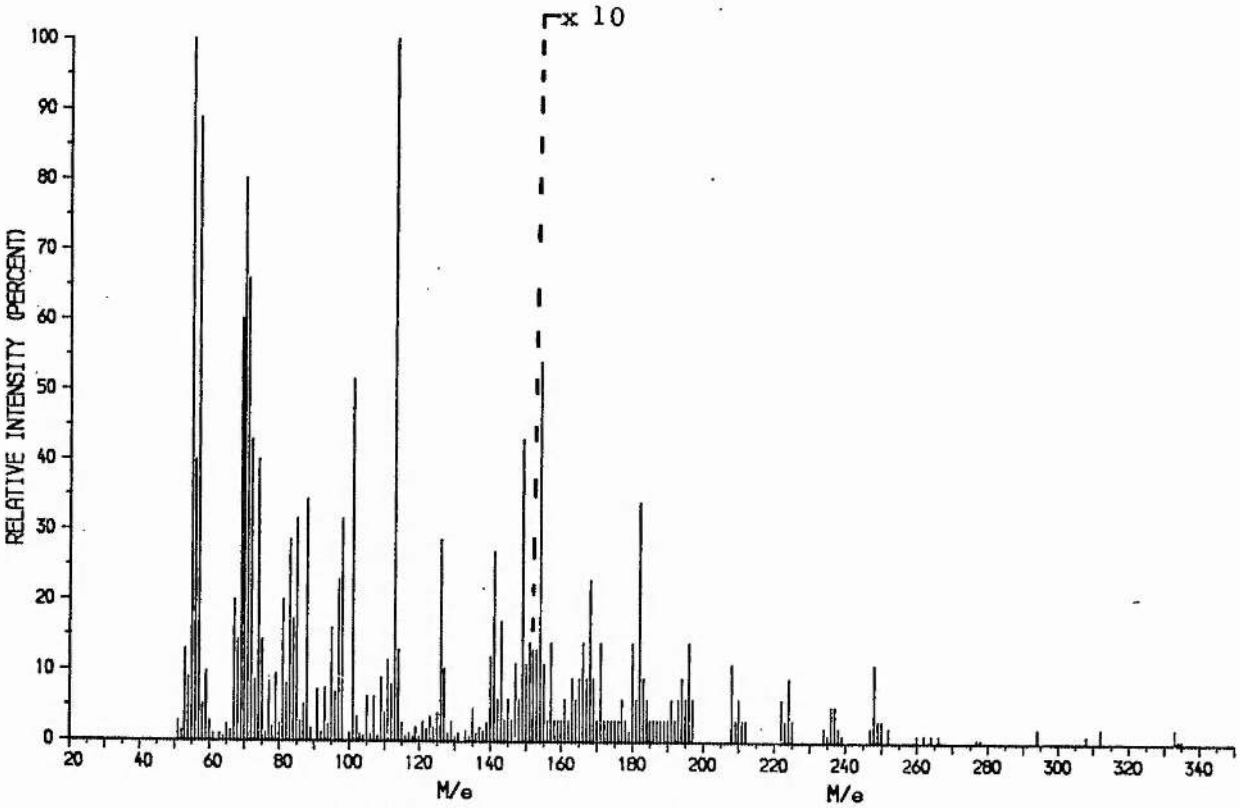
Mass spectrum 18. Methyl ester of C_{21} furanoid acid (F5)



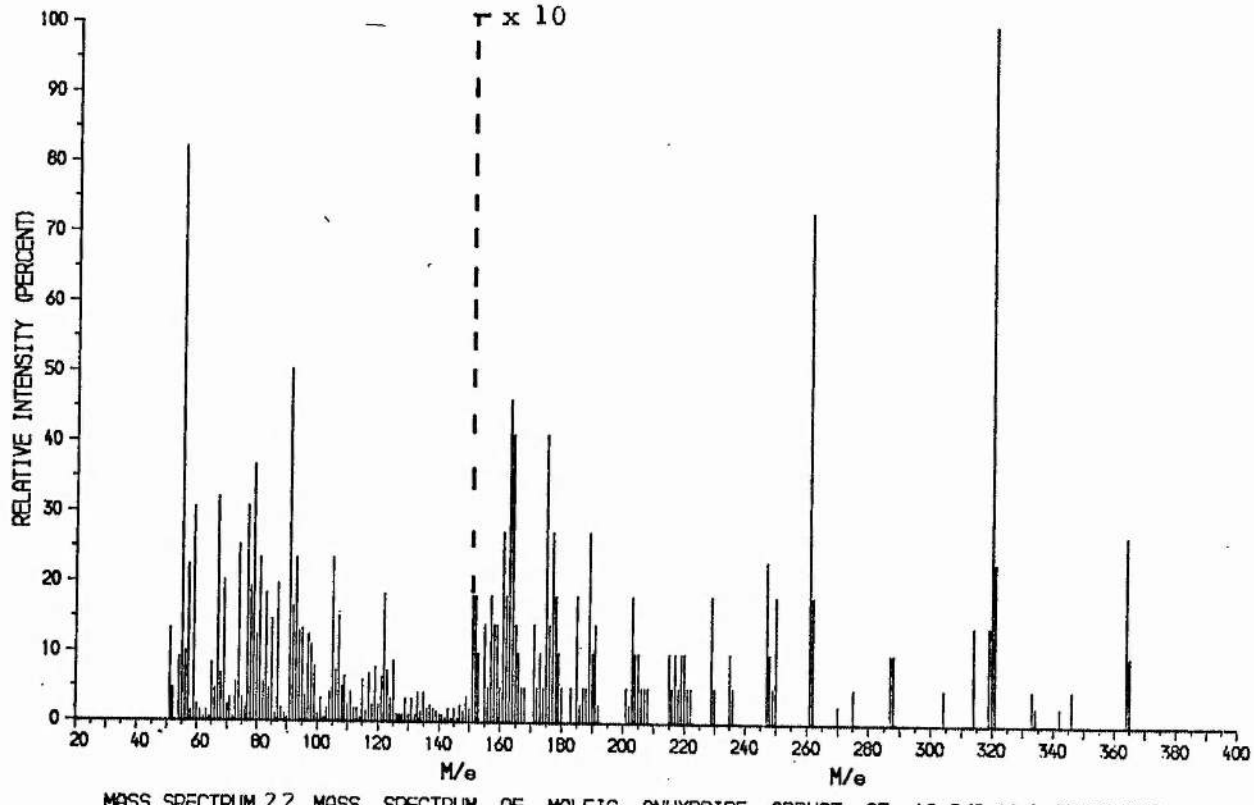
Mass spectrum 19. Methyl ester of C_{22} furanoid ester (F6)



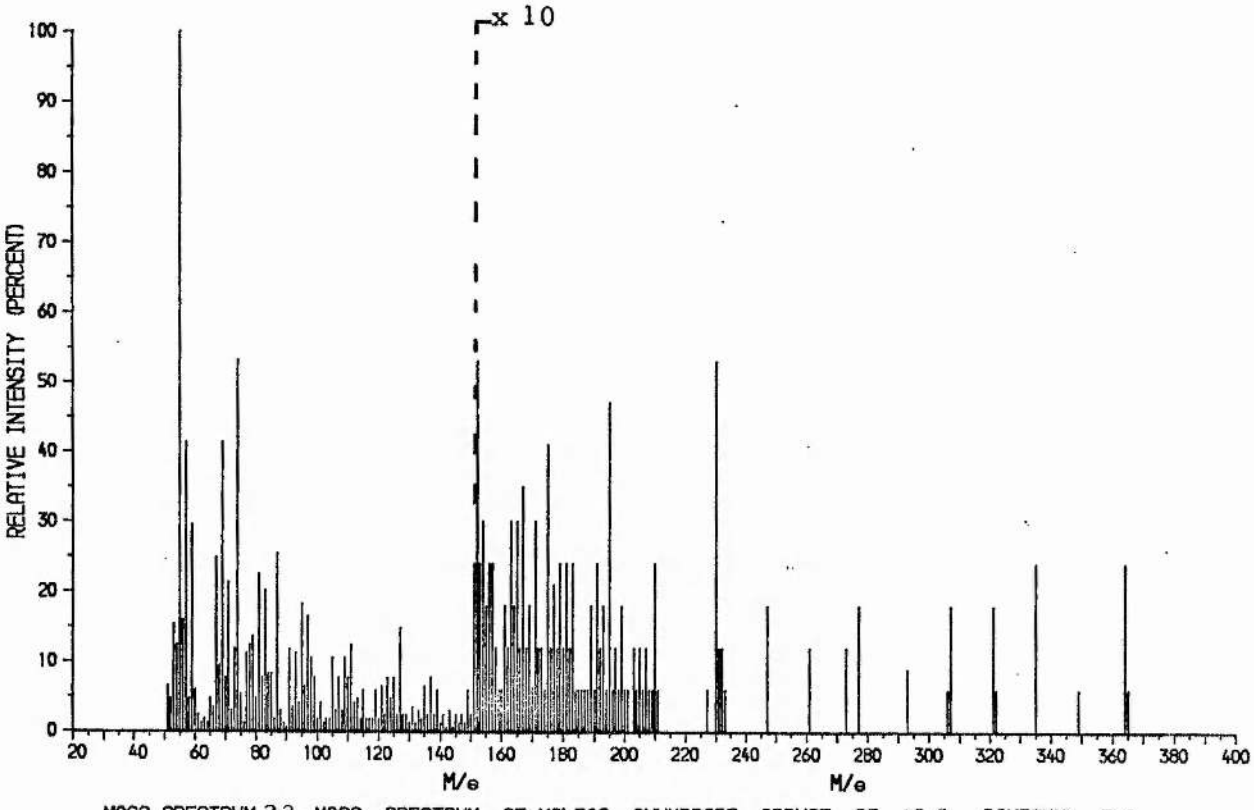
MASS SPECTRUM 20 MASS SPECTRUM OF HYDROGENATED U1 METHYL ESTER >85% PURITY



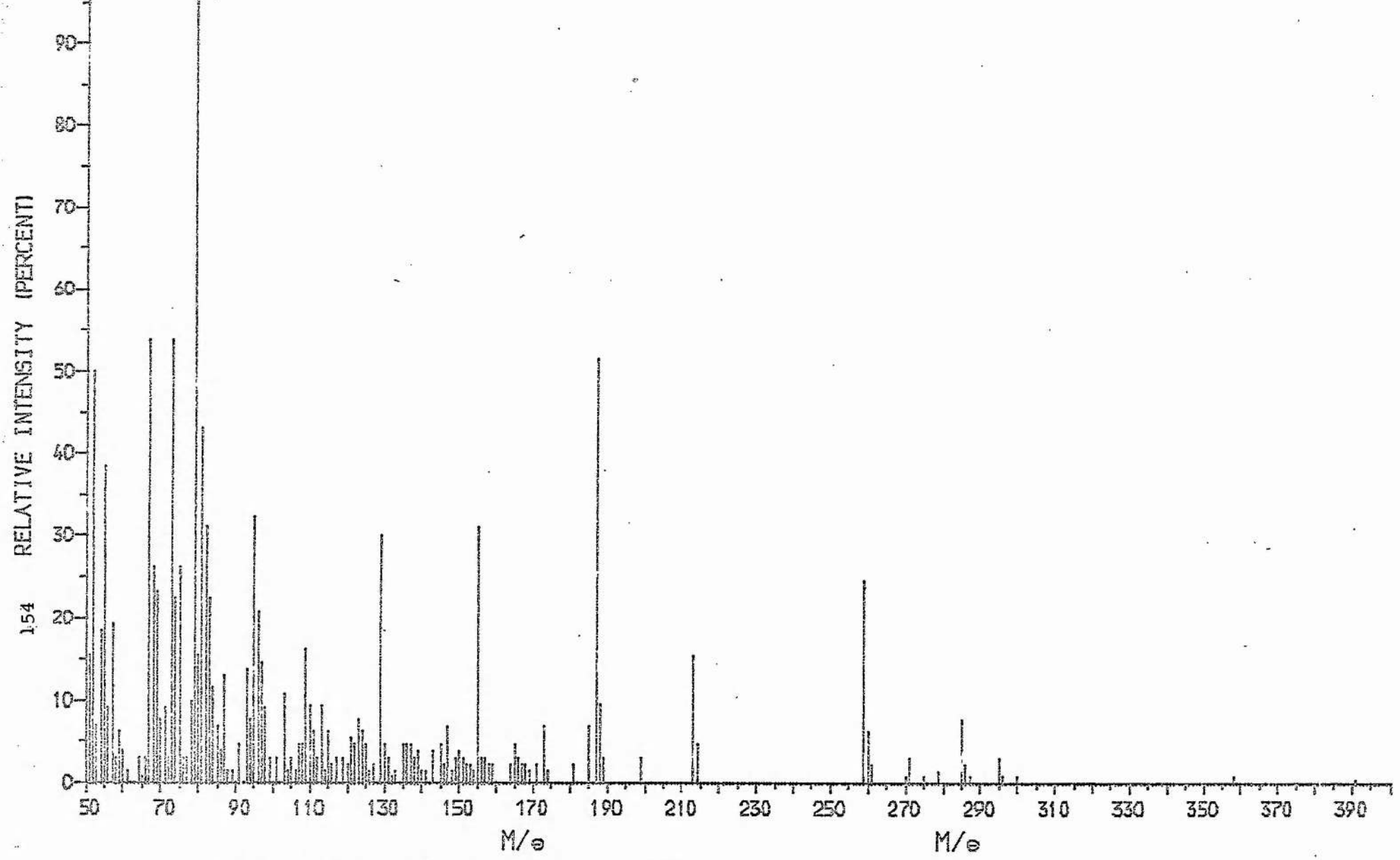
MASS SPECTRUM 21 MASS SPECTRUM OF 18-2 DIMETHYL 7,9 PYRROLIDIDE



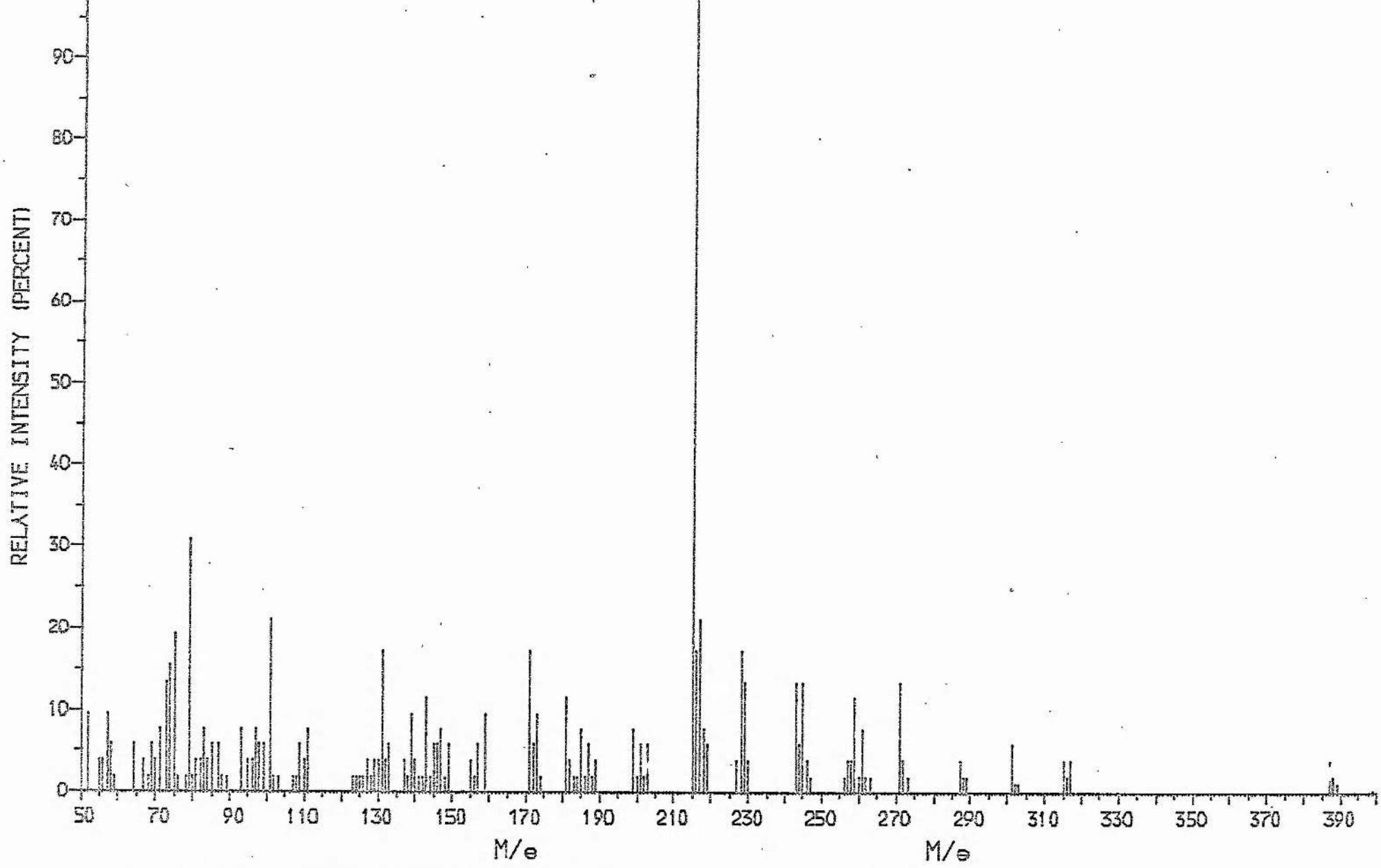
MASS SPECTRUM 22. MASS SPECTRUM OF MALEIC ANHYDRIDE ADDUCT OF 18-2(9,11) AUTHENTIC.



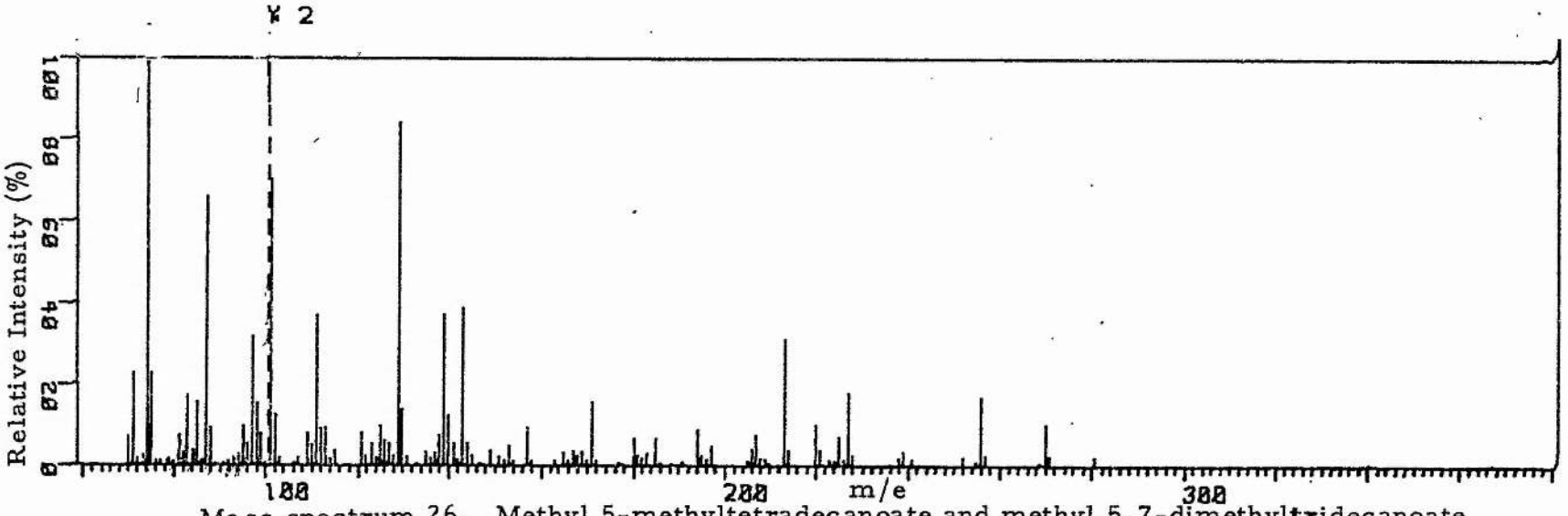
MASS SPECTRUM 23. MASS SPECTRUM OF MALEIC ANHYDRIDE ADDUCT OF 18-2 .DIMETHYL 7,9.



MASS SPECTRUM 24. OTMS DERIVATIVE OF 18:2(9,11) AUTHENTIC METHYL ESTER

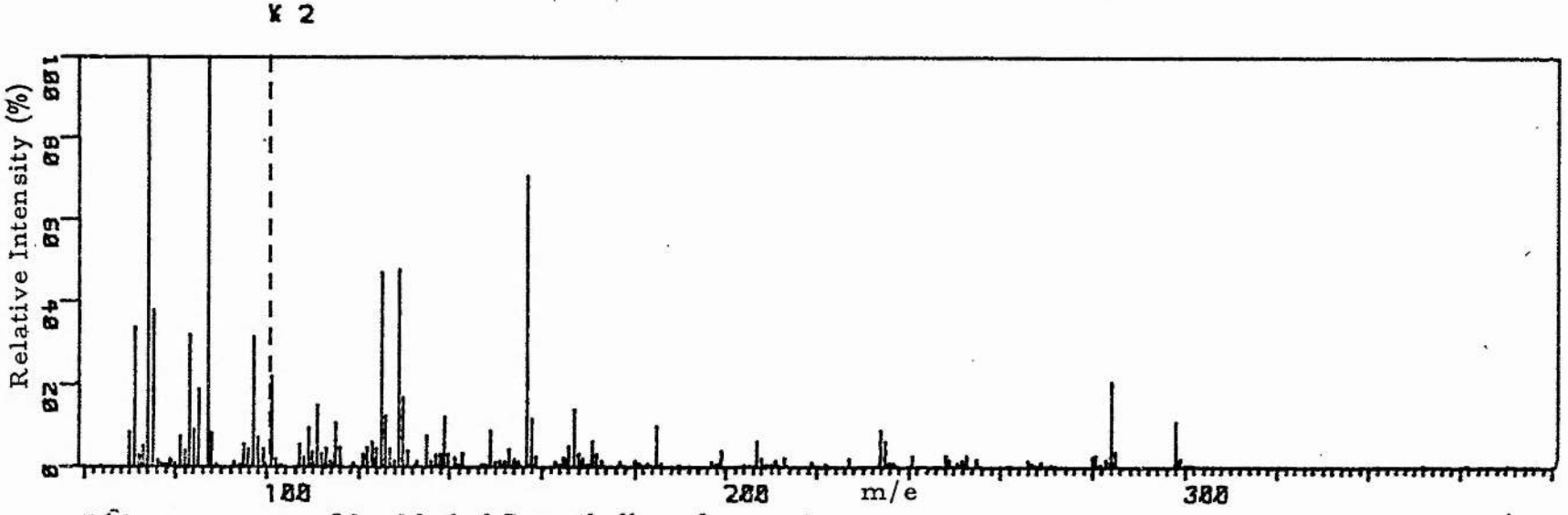


MASS SPECTRUM 25. OTMS DERIVATIVE OF U1, (7,9-DIMETHYLHEXADEC-6,8-DIENOIC ACID)

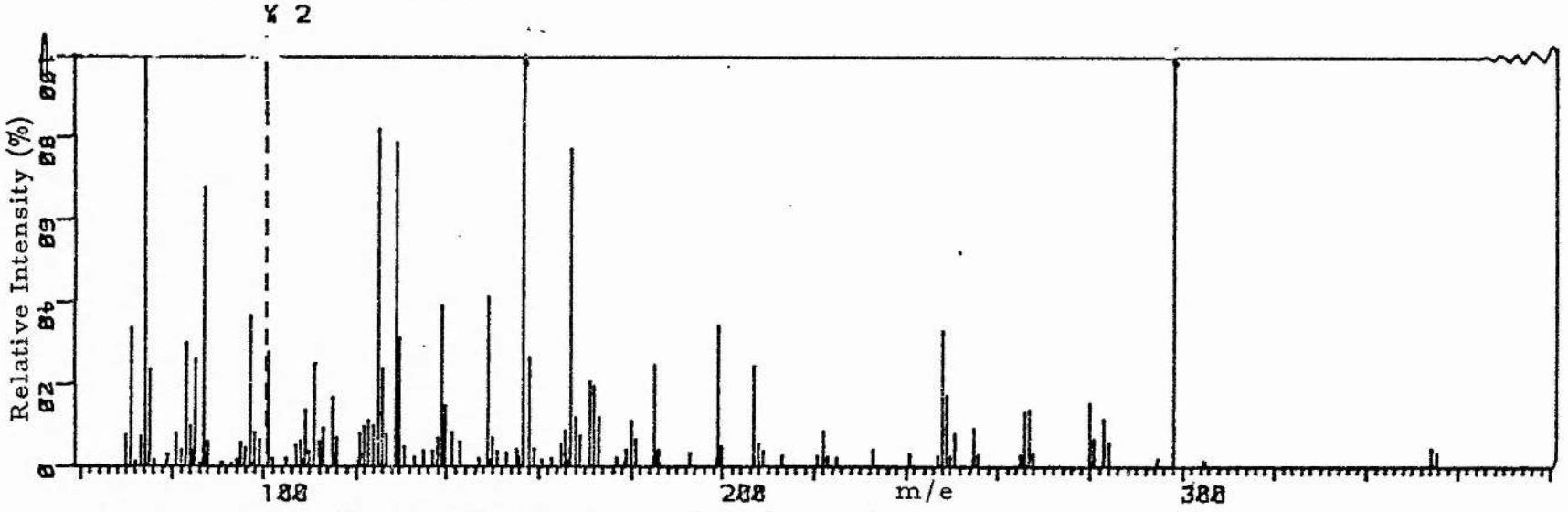


Mass spectrum 26. Methyl 5-methyltetradecanoate and methyl 5,7-dimethyltridecanoate

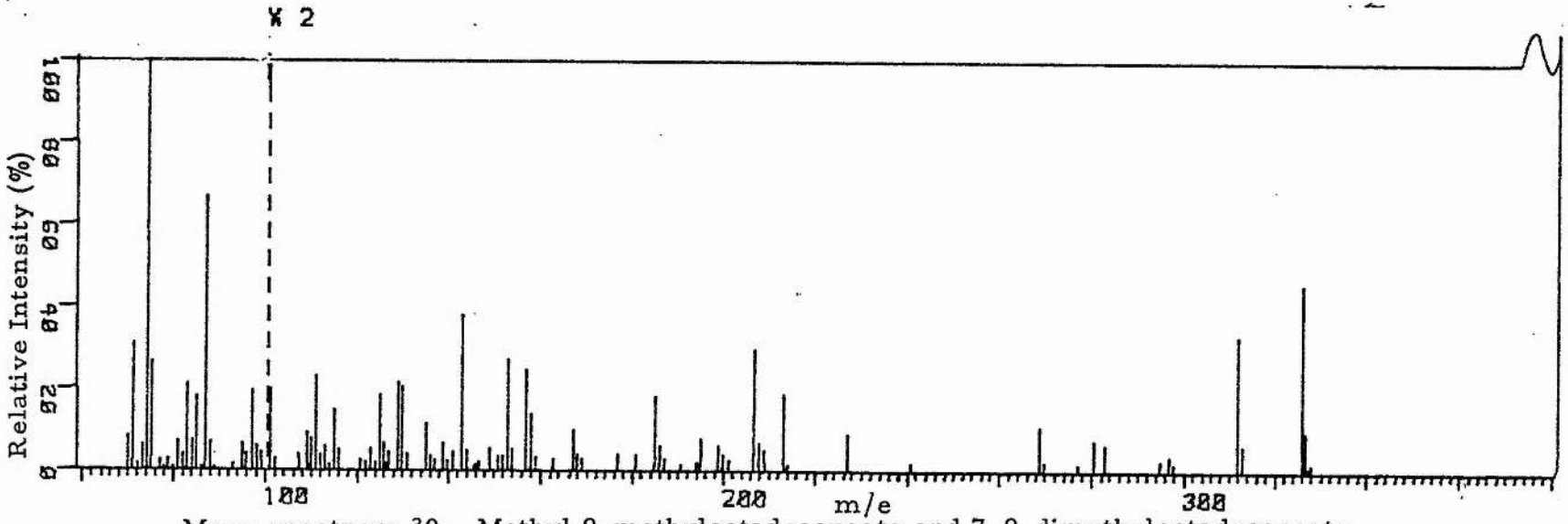
Relative Intensity (%)
100
80
60
40
20
0
x 2
100
200
300
m/e



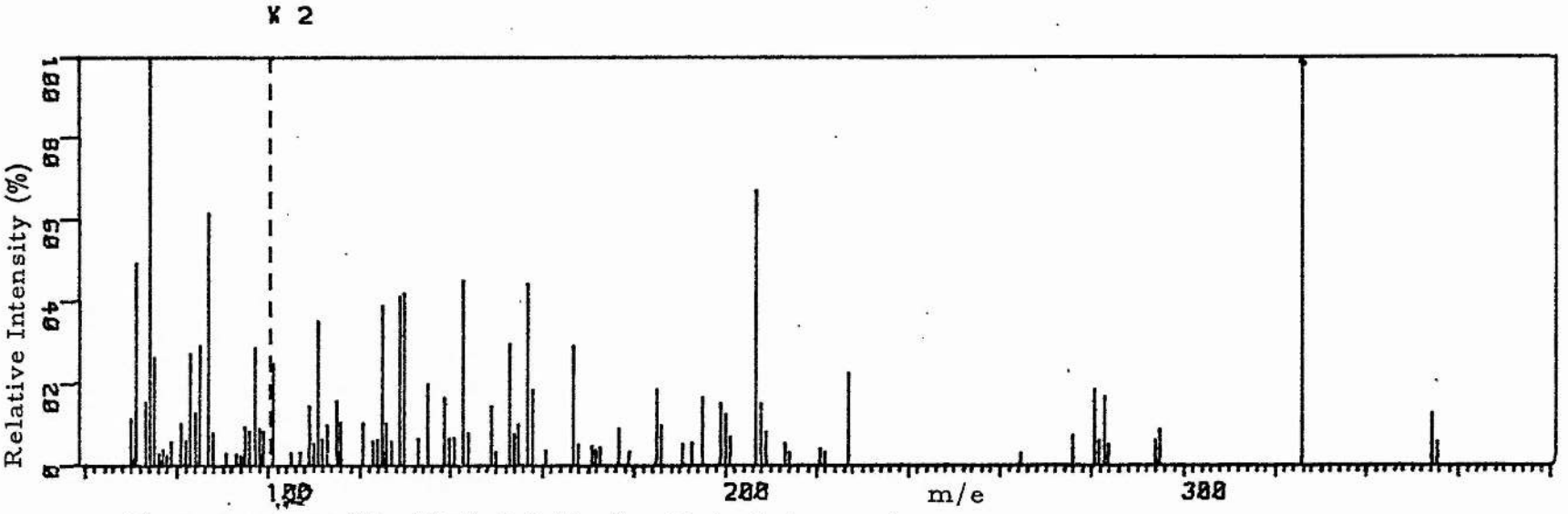
Mass spectrum 28. Methyl 7-methylhexadecanoate



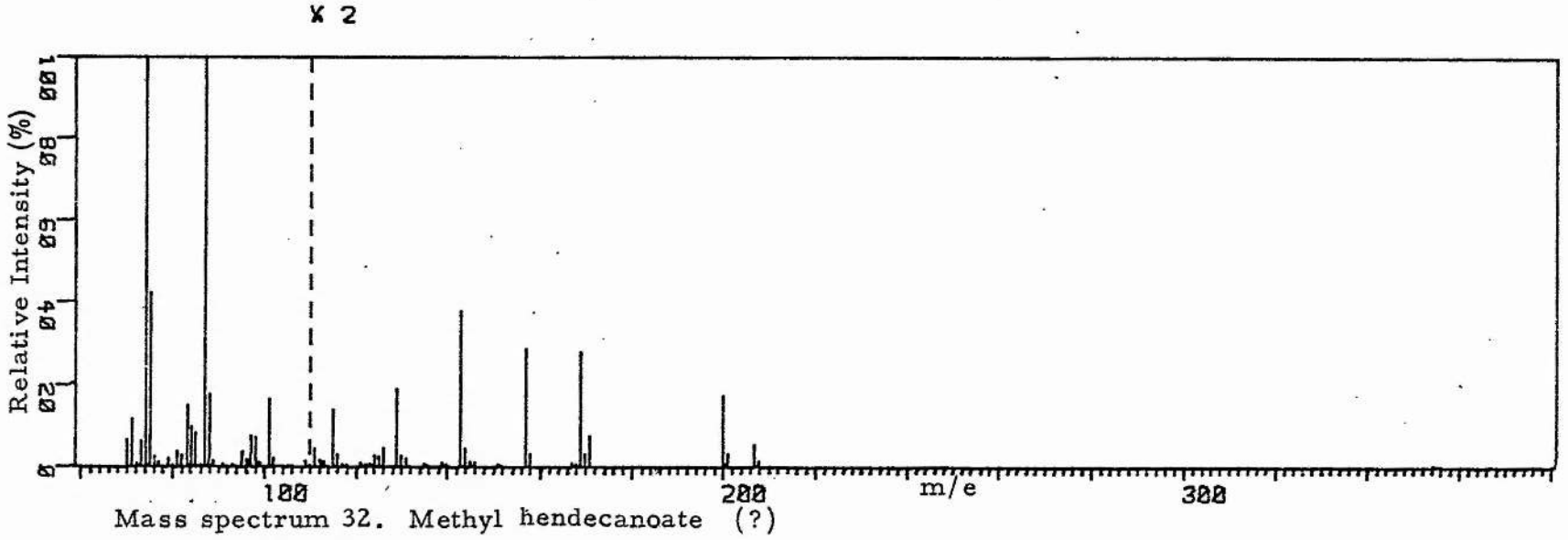
Mass spectrum 29. Methyl 7, 9-dimethylhexadecanoate



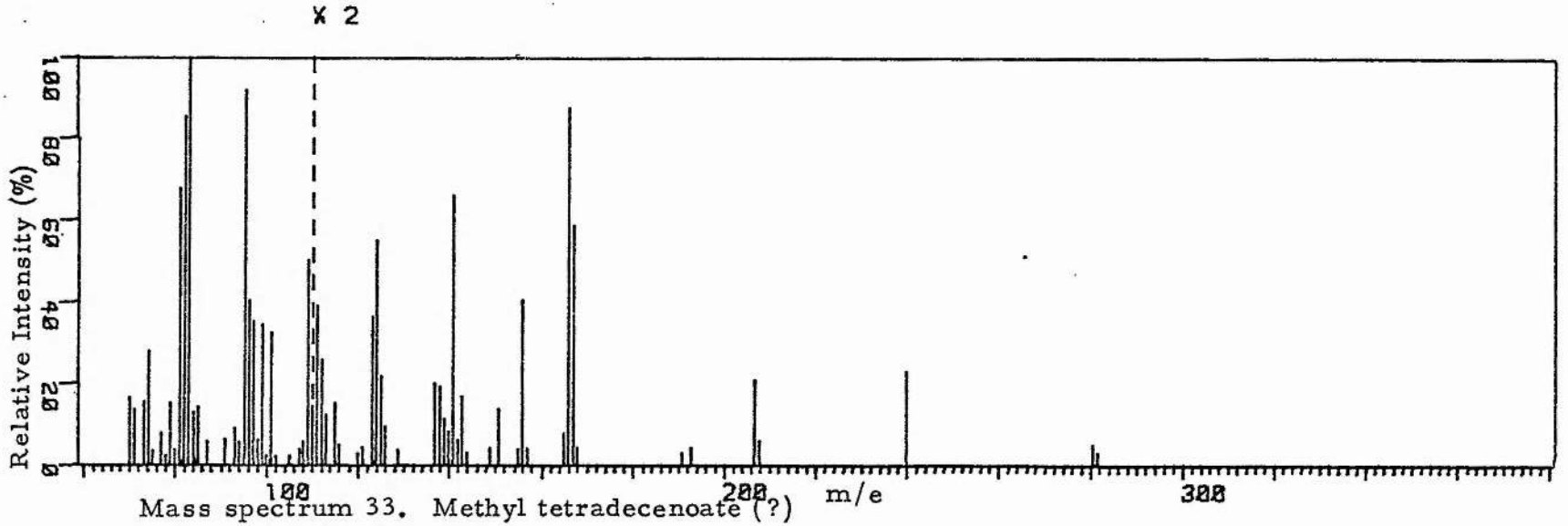
Mass spectrum 30. Methyl 9-methyloctadecanoate and 7,9-dimethyloctadecanoate



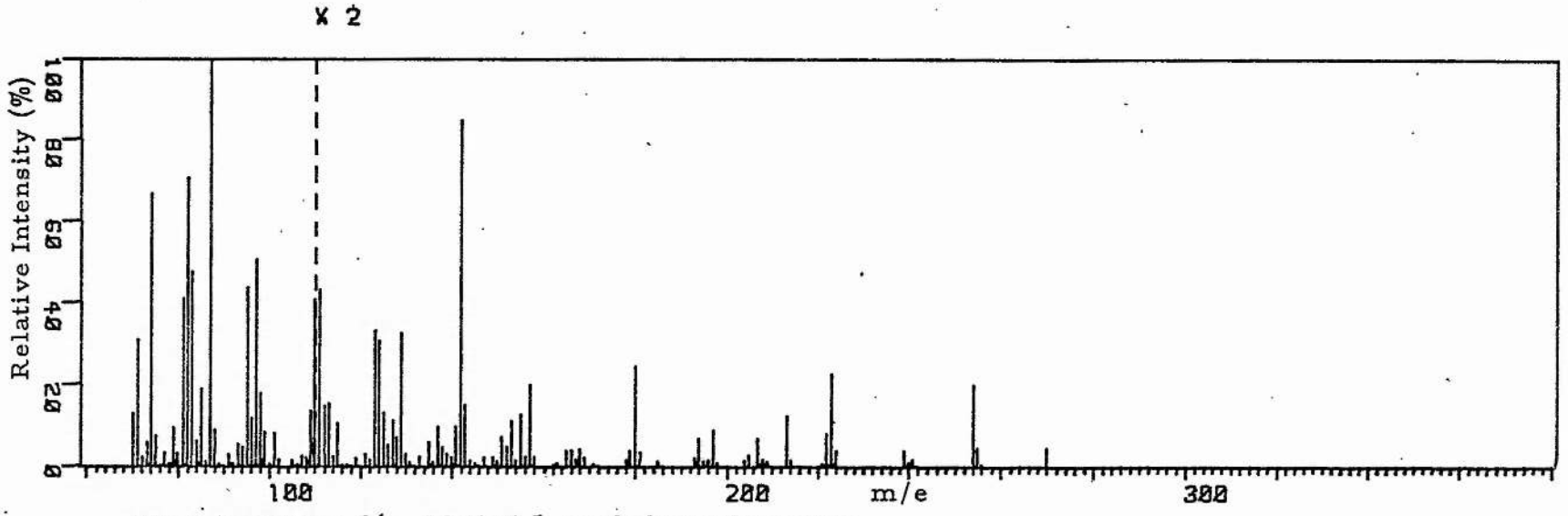
Mass spectrum 31. Methyl 9, 11-dimethyloctadecanoate



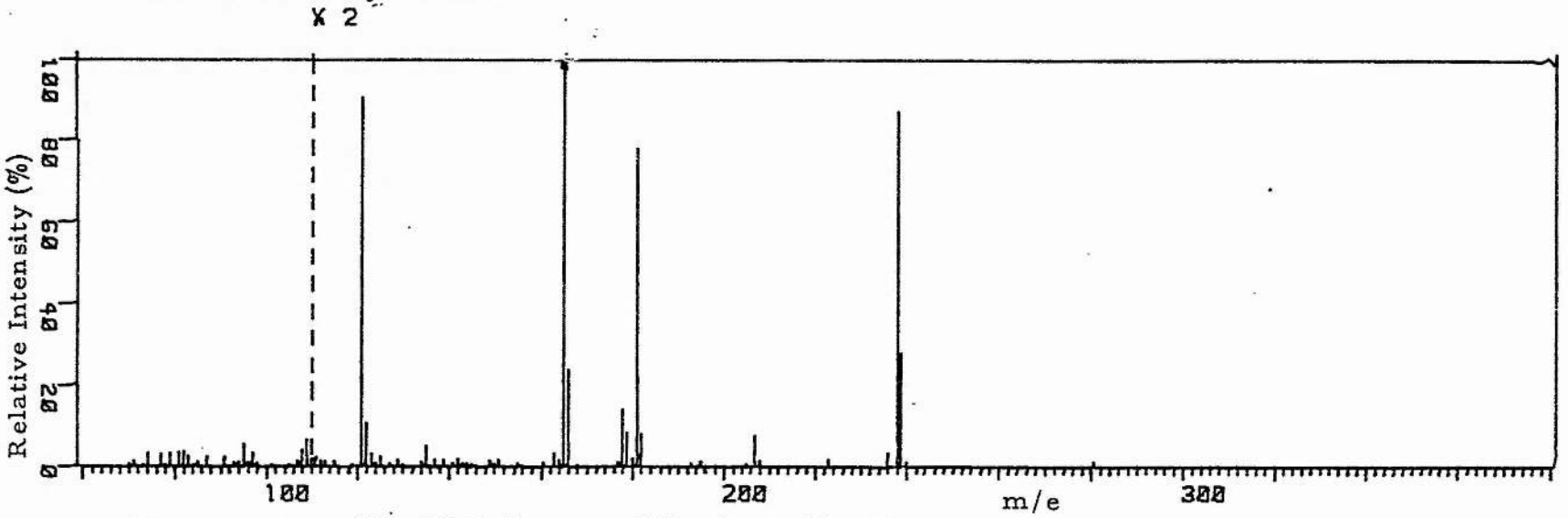
Mass spectrum 32. Methyl hendecanoate (?)



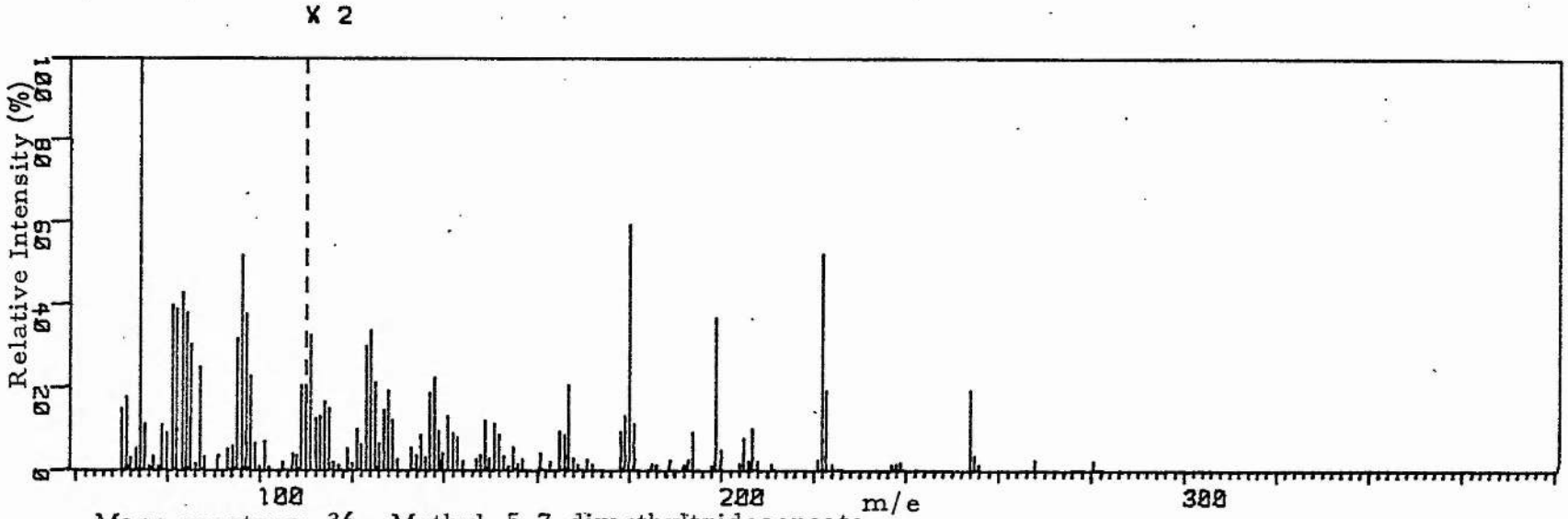
Mass spectrum 33. Methyl tetradecenoate (?)



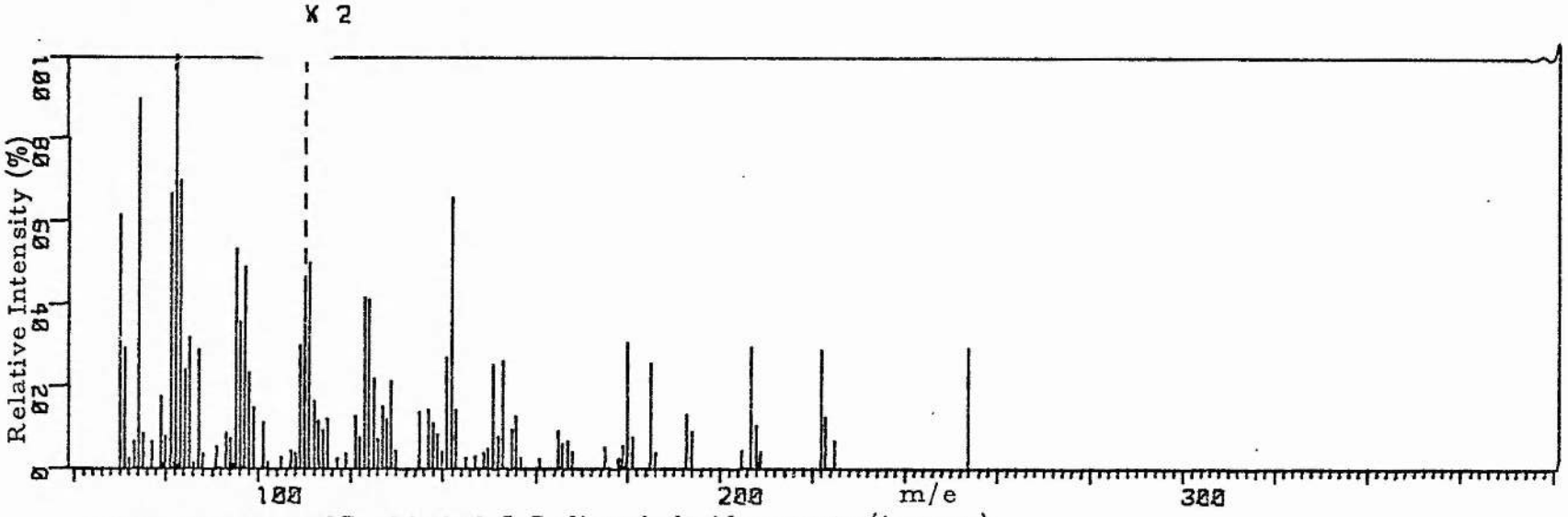
Mass spectrum 34. Methyl 5-methyltetradecenoate



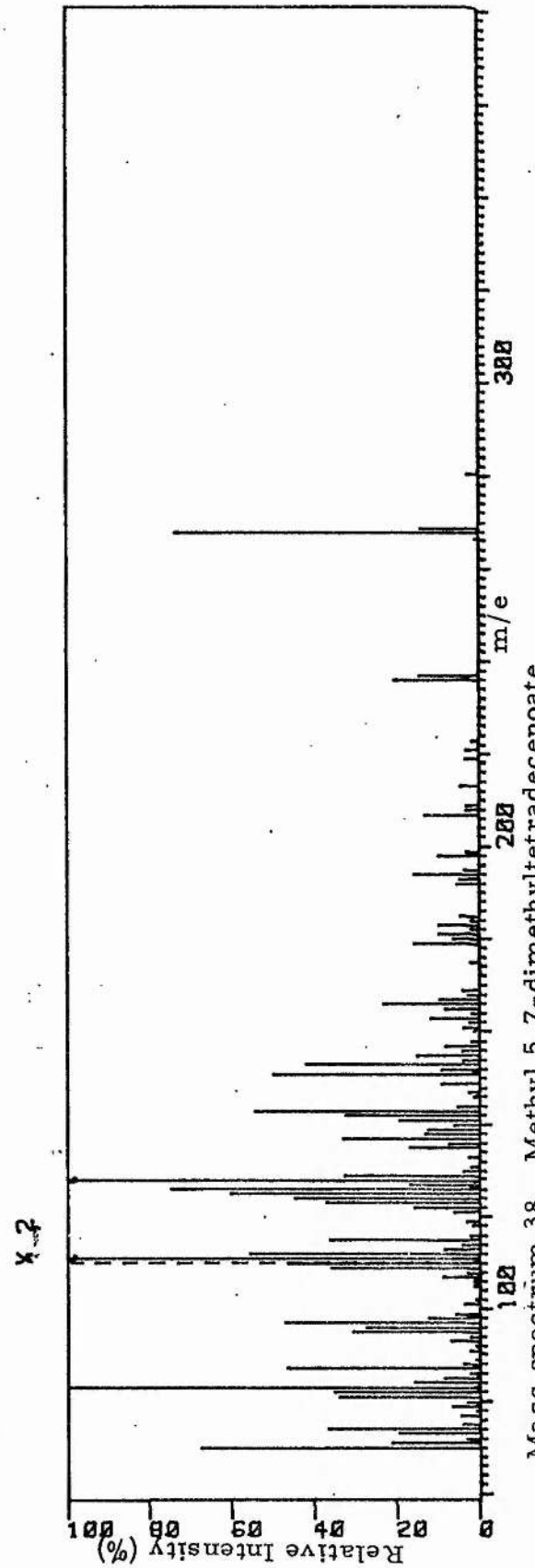
Mass spectrum 35. Methyl ester of C_{13} furanoid acid



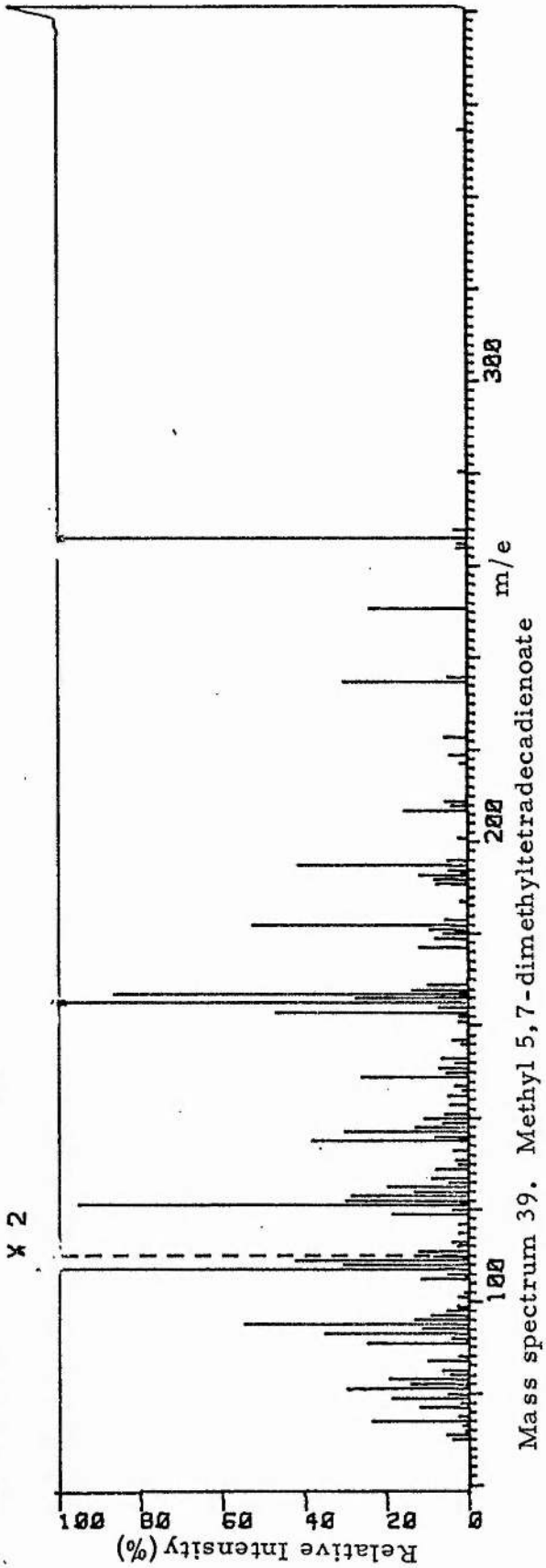
Mass spectrum 36 Methyl 5,7-dimethyltridecenoate



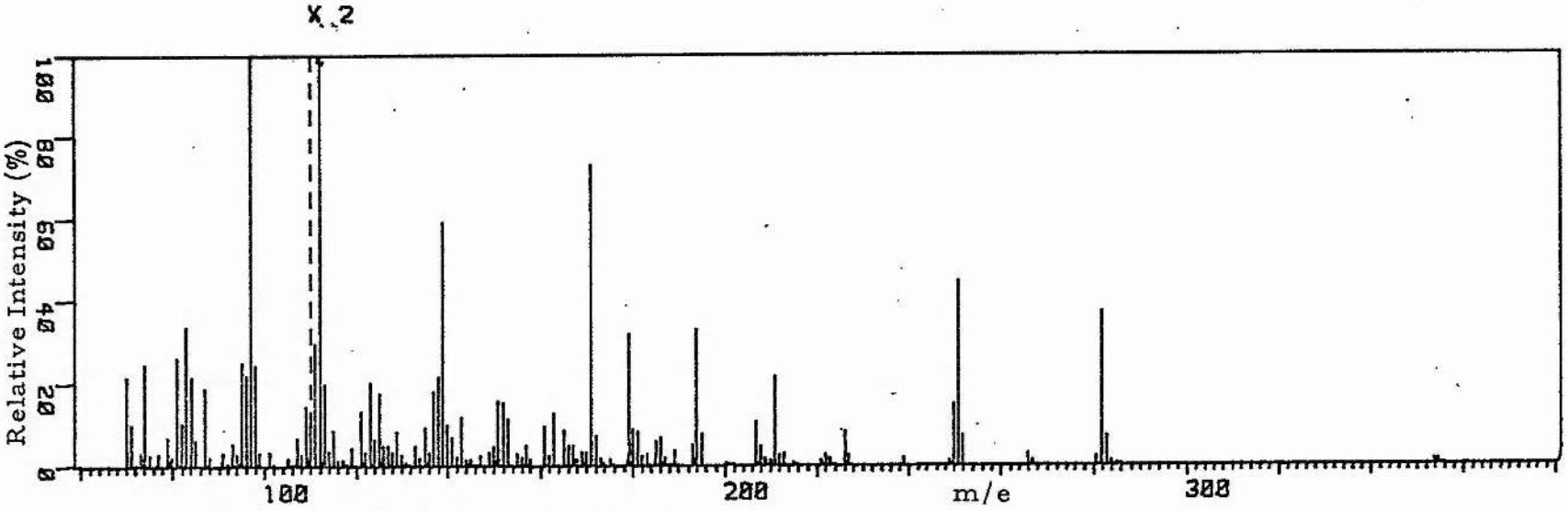
Mass spectrum 37. Methyl 5,7-dimethyltridecenoate (isomer)



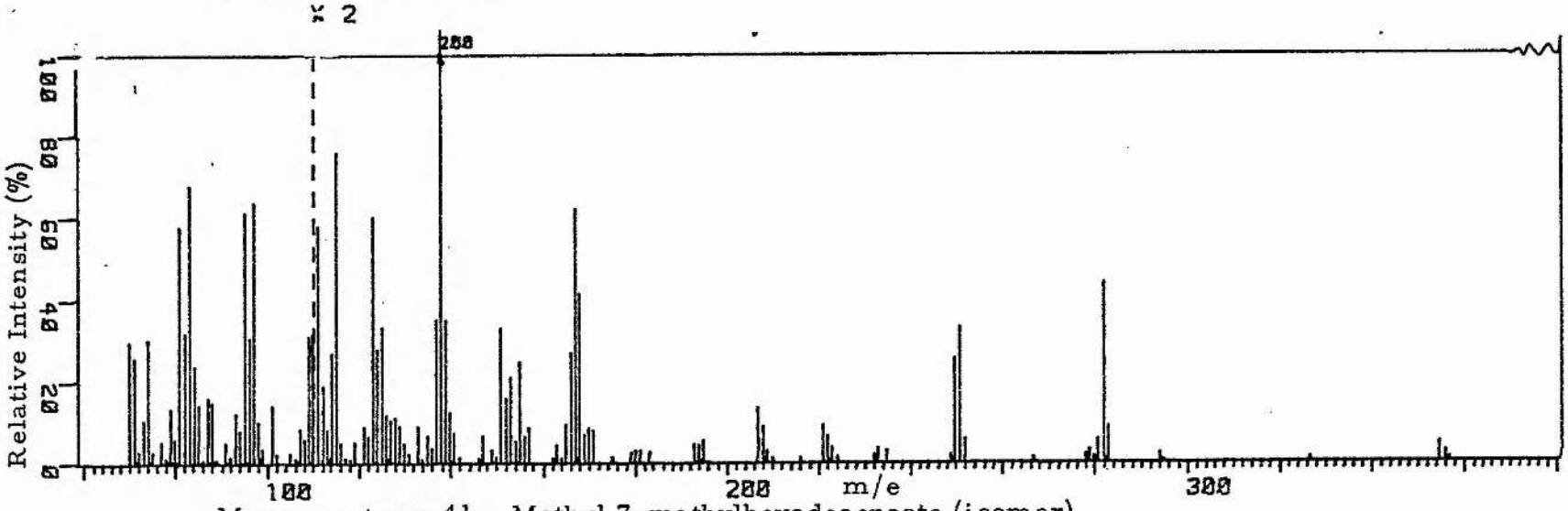
Mass spectrum 38. Methyl 5,7-dimethyltetradecenoate



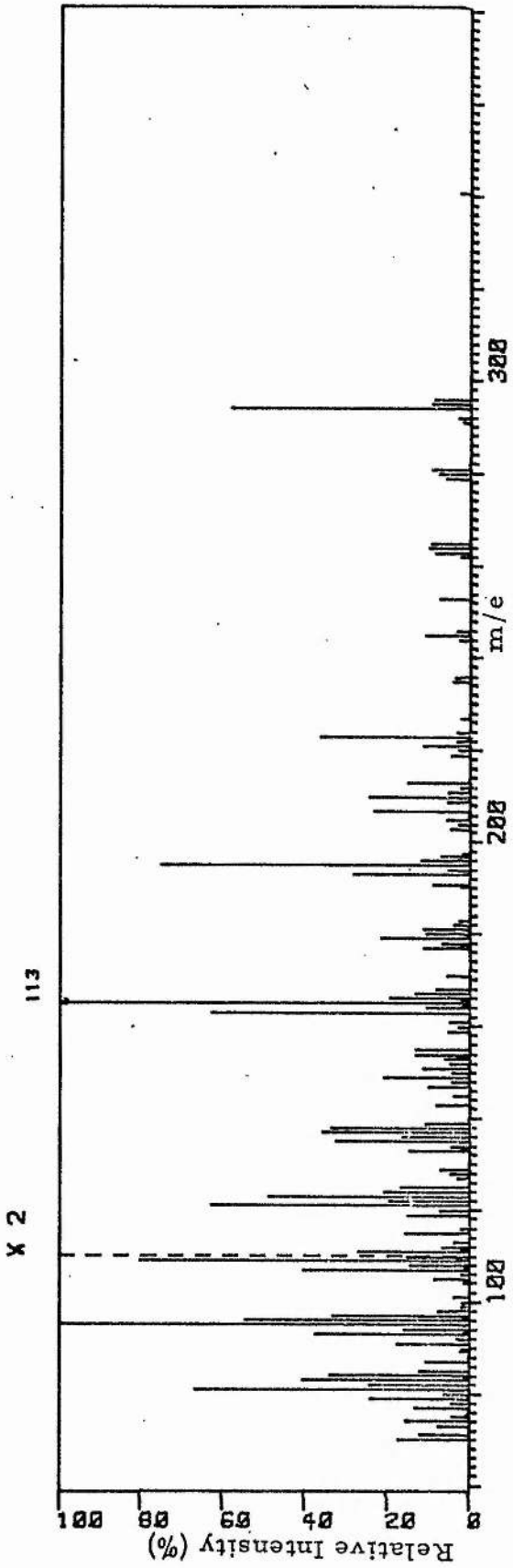
Mass spectrum 39. Methyl 5,7-dimethyltetradecadienoate



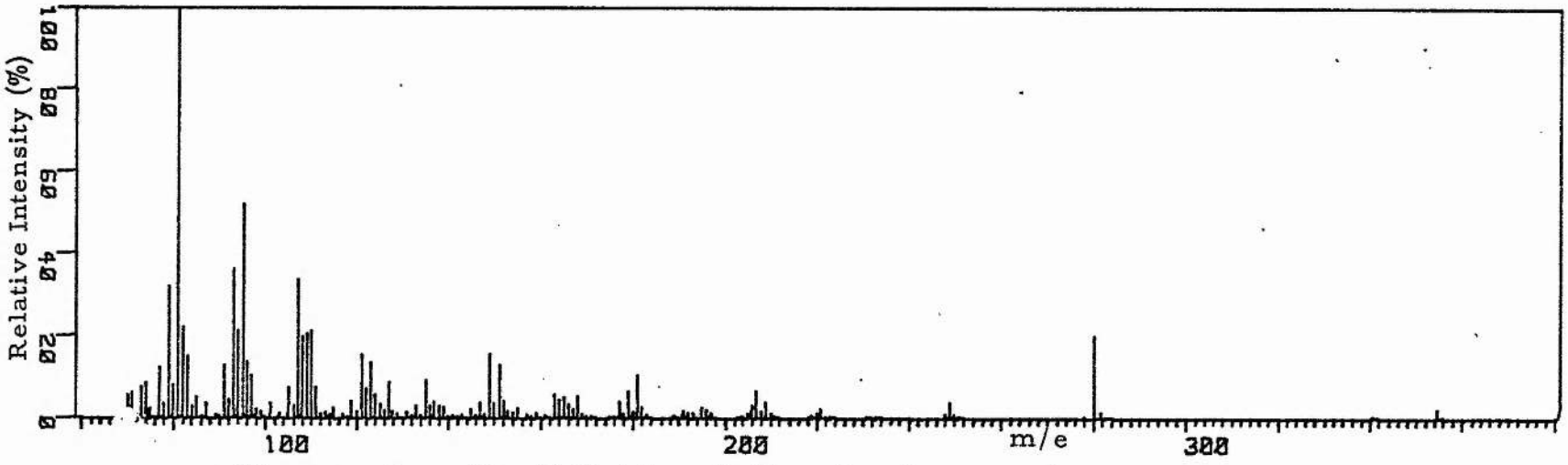
Mass spectrum 40. Methyl 7-methylhexadecenoate



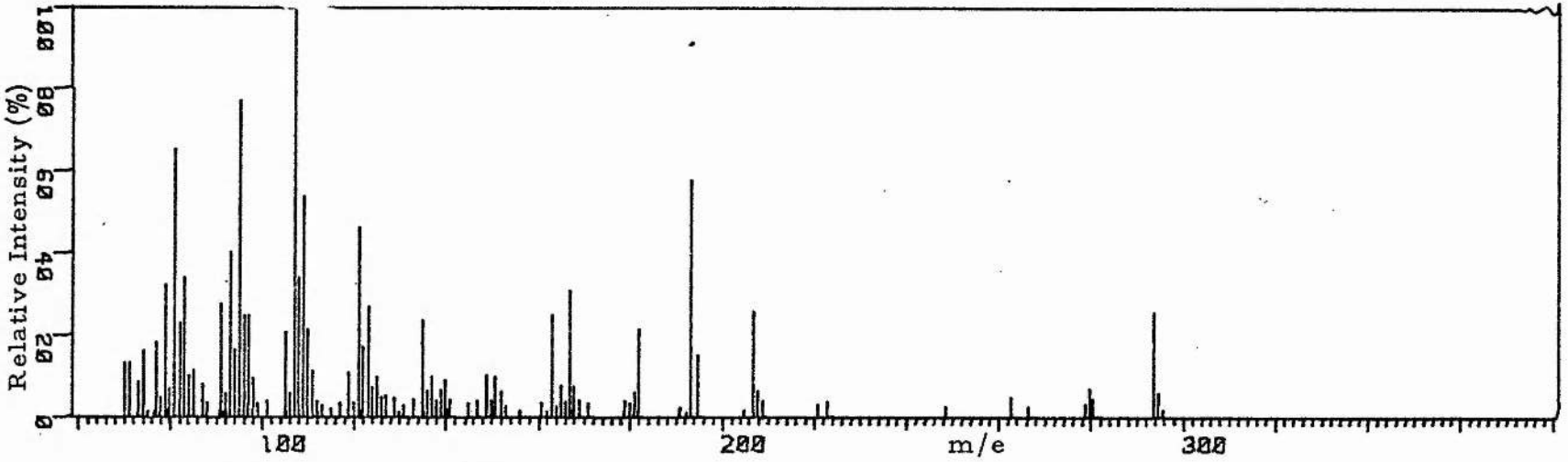
Mass spectrum 41. Methyl 7-methylhexadecenoate (isomer)



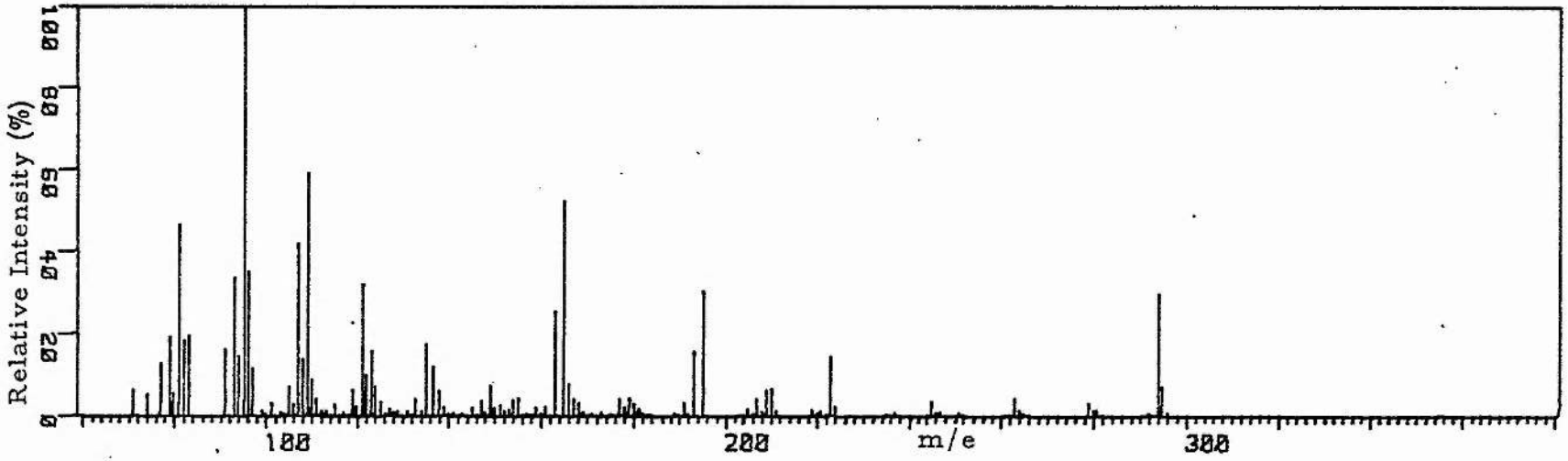
Mass spectrum 42. Methyl 7,9-dimethylhexadecadienoate



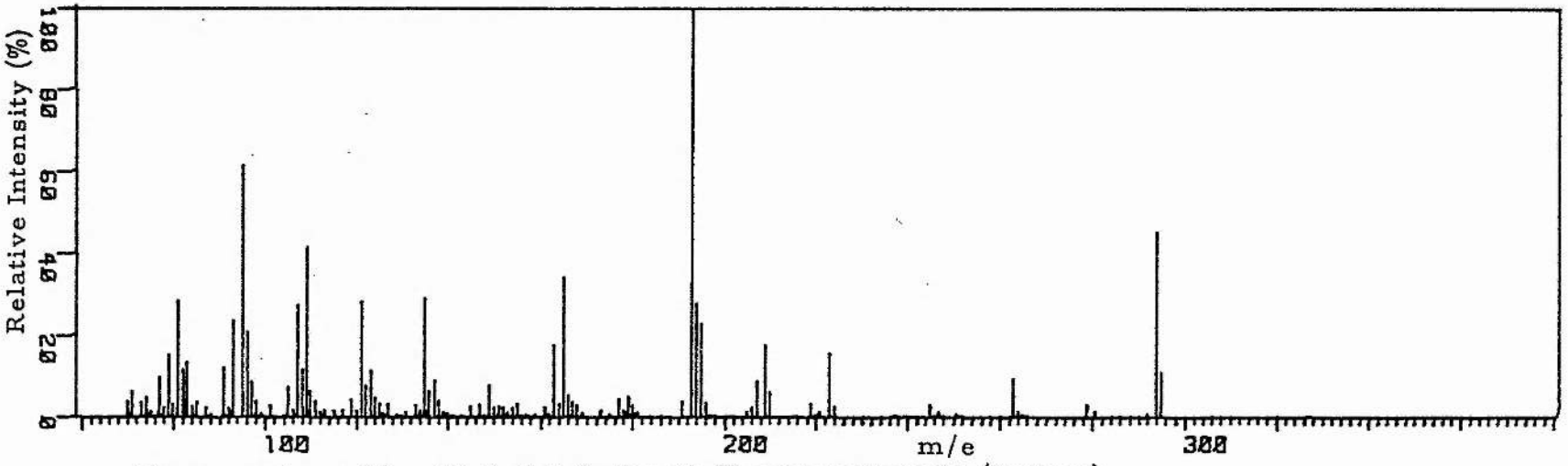
Mass spectrum 43. Methyl 7-methylhexadecadienoate



Mass spectrum 44. Methyl 7,9-dimethylhexadecadienoate (isomer)



Mass spectrum 45. Methyl 7, 9-dimethylhexadecadienoate (isomer)



Mass spectrum 46. Methyl 7, 9-dimethylhexadecadienoate (isomer)

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